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Monitoring the fate of metastatic tumor cells using magnetic cell tracking approaches (MRI and EPR)

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Abbreviations

^{18}F-FDG	18-fluorodeoxyglucose
^{18}F-HFB	Hexadecyl-4- ^{18}F fluorobenzoate
^{64}Cu-PTSM	^{64}Cu -pyruvaldehyde-bis-(N^4 -methylthiosemicarbazone)
$^{99\text{m}}\text{Tc}$-HMPAO	$^{99\text{m}}\text{Tc}$ -exametazime
^{111}In-oxine	^{111}In -oxyquinoline
AMPK	AMP-activated protein kinase
Au NPs	Gold nanoparticles
BCNU	Bischloroethylnitrosourea
BLI	Bioluminescence imaging
BM	Basal membrane
b-SSFP	Balanced steady-state free precession
BrdU	Bromodeoxyuridine
CA	Contrast agent
CAF	Cancer-associated fibroblast
CEST	Chemical-exchange saturation transfer
CLIO	Cross-linked iron oxide nanoparticles
CMCS	(carboxymethyl)chitosan
CNS	Central nervous system
CSC	Cancer stem cell
CSF-1	Colony stimulating factor-1
CT	Computer tomography
CTC	Circulating tumor cell
CTL	Cytolytic T lymphocyte
DMSA	Meso-2, 3-dimercaptosuccinic acid
DTC	Disseminated tumor cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPR	Electron paramagnetic resonance
EMT	Epithelial to mesenchymal transition
ESC	Embryonic stem cell
ESR	Electron spin resonance
fluc	Firefly luciferase

FION	Uniform ferrimagnetic iron oxide nanocubes
FGF	Fibroblast growth factors
FMT	Fluorescence-mediated tomography
GEM	Genetically engineered models of cancer
GFP	Green fluorescent protein
HA	Hemagglutinin
HSV1-tk	Herpes Simplex virus thymidine kinase 1
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IL-11	Interleukin-11
KSC	Kidney-derived stem cell
MAIL	Multiphoton absorption-induced luminescence
MEMRI	Manganese-enhanced MRI
MIRB	Molday Ion Rhodamine B [®] USPIO
MION	Monocrystalline iron oxide nanoparticle
MPI	Magnetic particle imaging
MPIO	Micron-sized iron oxides
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
NIR	Near-infrared
NMR	Nuclear magnetic resonance
PAI	Photoacoustic imaging
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PET	Positron emission tomography
PFC	Perfluorocarbon
PFCE	Perfluoro-15-crown-5-ether
PFOB	Perfluorooctylbromide
PFPE	Perfluoropolyether
PLL	Ploy- <i>L</i> -lysine
QD	Quantum dot
r₁	Longitudinal relaxivity
r₂	Transversal relaxivity

R₁	Longitudinal relaxation rate (1/T ₁)
R₂	Transversal relaxation rate (1/T ₂)
RF	Radiofrequency
ROS	Reactive oxygen species
SFB	<i>N</i> -succinimidyl 4-[¹⁸ F]-fluorobenzoate
SPECT	Single photon emission-computed tomography
SPIO	Superparamagnetic iron oxides
T₁	Longitudinal relaxation time
T₂	Transversal relaxation time
T_{2e}	Electronic transversal relaxation time
TAM	Tumor-associated macrophage
TfR	Transferrin receptor
TGF-β	Transforming growth factor-beta
TNM	Tumor, nodes, metastasis staging
UCNP	Upconversion nanoparticle
USPIO	Ultrasmall superparamagnetic iron oxides
VEGF	Vascular endothelial growth factor
VSOP	Very small superparamagnetic iron oxide particles

Foreword

Cancer is a leading cause of death worldwide. According to the World Health Organization (WHO), cancer was responsible for 7.6 million deaths in 2008 (~13% of all deaths). Moreover, projection studies estimate that deaths from cancer will continue to rise, with 13.1 million deaths in 2030. Numerous efforts have been made in fundamental research for understanding the characteristics of cancer. Despite these advances, cancer metastasis remains a bad prognosis event with uncharacterized features. The understanding of metastasis is rendered difficult by the extraordinary biological complexity of the process and the challenges for imaging the different steps of the metastatic cascade. The presence of metastasis dramatically affects the prognosis of patients. Improvements in cancer patients' survival are more likely due to recent progress in early diagnosis rather than break-through therapies. Still, the development of whole body imaging methods for elucidating the metastatic process in order to finally develop specific "anti-metastatic" drugs is needed.

On the other hand, cell-based therapies are promising strategies for curing several pathologies such as myocardial infarction, cartilage repair or neurodegenerative disorders. In this context, noninvasive cell tracking methods are used for monitoring the biodistribution of therapeutic cells. The aim of this thesis was to implement cell tracking for assessing the homing of metastatic cancer cells in distant organs. For such purpose, cells were first labeled *ex vivo* with iron oxide contrast agents, and imaged using magnetic resonance imaging (MRI). MRI has a high resolution and high soft-tissue contrast; the use of iron oxides improves its sensitivity. At the same time, iron oxides contained in tissues were quantified *ex vivo* using electron paramagnetic resonance (EPR). The contribution of EPR in MRI cell tracking studies represents another important part of the work. Besides imaging the fate of metastatic cells, information on their function/survival is important. Therefore, the last part of this work was focused on the metabolic consequences of an important step occurring during metastasis, i.e., the cell detachment.

The first part of the introduction aims to provide an overview of the "multistep" metastatic process. A brief description of the small animal models of metastasis is also included. The second part describes the noninvasive cell tracking methods for whole body imaging. Implications for cell-based therapies and cancer metastasis studies are also discussed. Basic principles of EPR and its use for iron oxide quantification are provided in the third part.

The aims and the results obtained in the course of the thesis are exposed in the second and third chapter. The first part of the study describes the use of EPR for assessing the yield of labeling with iron oxides. Next, a multimodal approach relying on bioluminescence imaging (BLI) and MRI cell tracking were used for monitoring the metastatic spread in different metastasis models. The third part of the study explores the complementarity of MRI and EPR for assessing the homing of brain metastases. The last part of the study describes the impact of cell detachment on oxygen consumption.

Finally, the main findings, the limitations and the perspectives of this work are exposed in the fourth chapter.

Chapter I: Introduction

I. Cancer metastasis

I.1. Definition

The term “metastasis” was first used by the French gynecologist Joseph Recamier in 1829, and can be defined as “the transfer of disease from one organ or part to another not directly connected to it” (Talmadge 2010). Metastasis is a hallmark of most malignant tumors and represents more than 90 % of the mortality associated to solid tumors (Gupta 2006). From a historical perspective, theories about the pathogenesis of metastasis have continuously been challenged. In 1889, the English surgeon Stephen Paget observed a discrepancy between the metastasis patterns and blood supply to organs based on autopsy records from 735 women suffering from breast cancer. He observed that breast cancer metastases preferentially developed in the liver, ovaries and bones (Talmadge 2010). Site-specific metastatic patterns are found in numerous cancer types as summarized in Table 1 (Nguyen 2009). The theory about the specific metastasis patterns was named “the seed and soil” hypothesis. Paget concluded that metastatic cancer cells (the seed) had a specific homing affinity to for some tissues (the soil) (Paget 1989). This theory, still prevailing nowadays, was challenged by Ewing and co-workers in 1928. Ewing thought that metastasis patterns were due to mechanical forces and the anatomical structure of the vascular network (Talmadge 2010). However, the seed and soil hypothesis was strengthened in 1980 by studies led by Hart and Fidler. Following the intravenous injection of B16 melanoma cells into mice grafted with ovary and kidney tissues under the muscle or subcutaneously, a preferential growth of melanoma cells in the lungs and in the ovary tissues but not in the kidney was observed (Hart 1980). It is currently accepted that mechanical factors are not sufficient to drive the site-specific establishment of metastasis. Depending on the location, endothelial cells express different adhesion molecules and some cancer cells express corresponding ligands (e.g., integrin $\alpha_4\beta_1$ expressed by B16F10 melanoma binding to VCAM-1 expressed by endothelial cells) (Bendas 2012). This results in a specific arrest of metastatic tumor cells in a given tissue. Second, chemokines play a pivotal role in the site-specific arrest of cancer cells. For instance, CXCR4 receptor is overexpressed in many breast cancer cell types. The corresponding ligand, SDF-1/CXCL12 is highly expressed in lymph nodes and in the lungs. By using anti-CXCR4-antibodies, Müller *et al.* succeeded to impair lung metastasis in immunodeficient mouse xenograft experiments (Muller 2001).

Table 1: Typical sites of metastatic relapse for solid tumors (Nguyen 2009).

Tumor type	Principal sites of metastasis
Breast	Bones, lungs, liver and brain
Lung adenocarcinoma	Brain, bones, adrenal gland and liver
Skin melanoma	Lungs, brain, skin and liver
Colorectal	Liver and lungs
Pancreatic	Liver and lungs
Prostate	Bones
Sarcoma	Lungs
Uveal melanoma	Liver

I.2 The metastatic cascade

For the sake of clarity, only tumors arising from epithelial tissues (carcinomas) will be discussed in this section. Carcinomas represent ~80 % of life-threatening cancers (Valastyan 2011). It is currently known that not all cancer cells within a primary tumor have the capability to form metastasis. The cancer cell population is highly heterogeneous and only a subset of cells has self-renew traits. Such population, known to encompass cancer stem cells (CSC), is found in human acute myeloid leukemia as well as in solid tumors including breast cancer or colon cancer (Bonnet 1997, Al-Hajj 2003, O'Brien 2007, Coghlin 2010). During the metastatic process, CSCs are exposed to multiple stresses such as hypoxia, inflammation, pH changes and nutrient deprivation. Although these stresses constitute hurdles for tumor growth, they also provide a selection pressure promoting the development of aggressive phenotypes (Mendoza 2009). Metastasis is described as a multistep process. Cancer cells may directly spread via blood vessels or disseminate to regional lymph nodes via the lymphatic vessels. In comparison to hematogenous metastasis, lymphatic metastasis is considered as a passive mechanism because lymphatic vessels lack mechanical barriers such as inter-endothelial cell tight junctions, pericytes, and basal membranes (Joyce 2009). The lymphatic dissemination of cancer cells is a prognostic marker because it reflects the invasiveness of the tumor. Hematogenous dissemination is, however, considered as the main mechanism of metastatic dissemination (Gupta 2006). The main steps of hematogenous dissemination of cancer cells are shown in Figure 1.

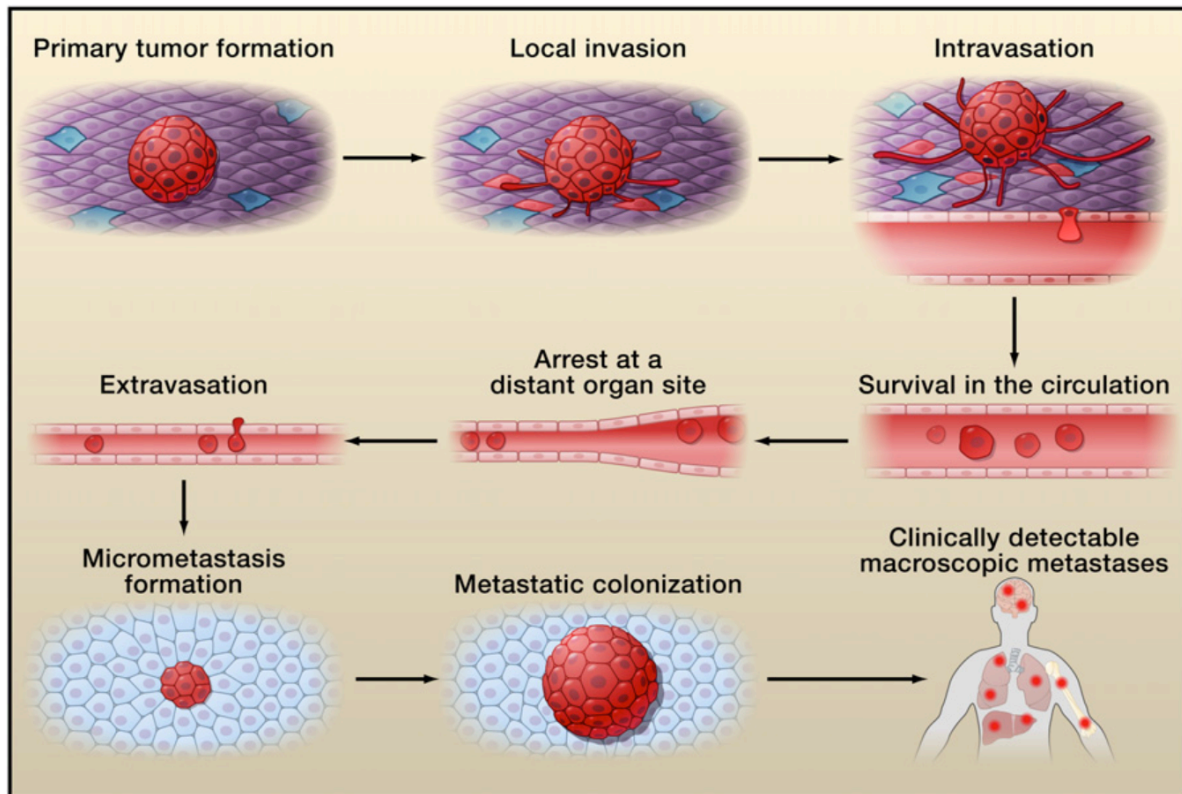


Figure 1: Main steps of metastatic progression (Valastyan 2011).

1. Local invasion of tumor cells through basal membrane degradation and extracellular matrix remodeling.
2. Intravasation into the lumina of blood vessels.
3. “Life in transit”, circulation of cancer cells in the blood stream.
4. Arrest at a distant site.
5. Extravasation into the parenchyma of foreign tissues.
6. Survival and micrometastasis establishment.
7. “Metastatic colonization. Reinitiation of a proliferative program leading to the formation of macrometastases.

Of note, the course of metastasis is tumor-dependent. For instance, oestrogen receptor-positive breast tumors are known to infiltrate distant sites early in the disease. Nevertheless, the latency period is generally long (several months to years). This implies that disseminated tumor cells are not fully competent to colonize distant organs once they leave the primary tumor. On the other hand, the latency period is short (months) for lung and pancreatic adenocarcinomas. This means that in these cases cancer cells acquire sufficient malignant traits in the primary tumor to efficiently infiltrate and colonize distant organs. In other words, these cells are already well adapted to metastasize once they leave the primary tumor. Finally, the development of a primary tumor is very long for colorectal carcinoma (decades). But once

the invasive stage is reached, infiltration and colonization of the liver and the lungs (less frequent) by cancer cells quickly occurs (Nguyen 2009).

In clinical situations, metastasis is often considered as being a late process occurring in cancer patients with advanced diseases. This paradigm, known as the linear progression model, is at the basis for tumor, nodes, metastasis staging (TNM). There is indeed a correlation between tumor size and metastatic risk. For instance, the median time from tumor resection to a diagnosis of metastasis for patients with a T1 (<2 cm) tumor is 35 months versus 19.9 months for patients with a T3 (>5 cm) tumor. Such observation supports the linear progression model (Engel 2003, Talmadge 2010, Sethi 2011). On the other hand, metastatic cells can spread early after the onset of the disease. It was shown that breast cancer cells could disseminate from pre-neoplastic lesions and infiltrate the bone marrow and other tissues (Coghlin 2010, Hanahan 2011). This paradigm, known as the parallel progression model, is strengthened by the observation that about one third of breast cancer patients with no sign of metastasis are found with disseminated cancer cells in the bone marrow (Hedley 2009).

1.2.1. Local invasion

During carcinogenesis, benign hyperplasia progresses to the formation of a carcinoma *in situ* to finally become a locally invasive carcinoma. Genetic and morphological changes occur during carcinogenesis. The incidence of mutations increases with the progression of the disease and supports a clonal model of carcinogenesis. Mutations result in the constitutive activation within CSCs of pro-survival pathways (oncogenes) in concert with the down-regulation of tumor suppressor genes. Transformed cells acquire malignant traits and start to detach from the epithelial sheet. When detached from the basal membrane (BM), normal epithelial cells die via a specialized apoptotic process called “anoikis” (homeless in Greek) (Gillies 2008). This mode of cell death is mandatory under physiological conditions to prevent the proliferation of cells in unwanted areas. Anoikis is triggered by the loss of anchorage of cells to substratum and involves integrin signaling. Under normal circumstances, integrins form adhesion complexes with components of the extracellular matrix (ECM). When cells are detached, α and β subunits of integrin heterodimer receptors are disrupted. It results in the activation of the death receptor (caspase 8 activation in a FADD-dependent manner) and/or mitochondrial pathways of caspase activation (Simpson 2008, Horbinski 2010). Acquired anoikis resistance allows cancer cells to survive after detachment from the ECM. It was shown however that inhibition of apoptosis alone was not sufficient to prevent cell death after detachment (Mailleux 2007). Once detached from the

extracellular matrix, metabolic changes occur and cells undergo energy deprivation. Glucose uptake is decreased, affecting both glycolysis and the pentose phosphate pathway. As direct consequences, ATP production via the tricarboxylic acid cycle is significantly decreased and the antioxidants defenses are altered. Furthermore, reactive oxygen species (ROS) levels are increased in detached cells and prevent ATP production from fatty acid oxidation. This starvation mechanism leads to cell death (Gottlieb 2009). Schafer *et al.* showed that activation of oncogenes, namely ErbB2 (also known as Her2), restored glucose uptake and promoted survival of detached breast cancer cells (Schafer 2009b).

The proliferation of epithelial cells is strictly controlled by cell-cell (an adjacent cell must die and be released in the lumen) and cell-BM interactions (Gillies 2008). One of the key molecular actors of contact inhibition of proliferation is the epithelial cadherin or E-cadherin (McClatchey 2012). This protein is involved in cell-cell junctions where it helps to maintain the integrity of epithelial layers. Escaping these regulatory mechanisms is thus mandatory to pursue invasion. E-cadherin is one of the critical elements which orchestrates the organization of the epithelial sheets. Cancer cells may co-opt a cell biological process known as the “epithelial to mesenchymal transition” (EMT) to overcome this obstacle (Valastyan 2011). The EMT program occurs during embryogenesis, wound repair, and cancer progression (Miyazono 2012). In cancer, the EMT paradigm emerged on the basis that the progressive loss of the E-cadherin expression was associated with an invasive and metastatic phenotype. The EMT defines the process during which transformed epithelial cells lose the expression of tight and adherens junctions. Cell polarity is lost and cells differentiate into mesenchymal-like cells. EMT program in cancer cells leads to the acquisition of abilities to invade, to resist apoptosis, and to disseminate. Most of the molecular actors involved in EMT, such as the transcription factors Snail, Slug, Twist, and Zeb1/2, are involved in migratory processes occurring during embryogenesis. Activation of these transcription factors leads to the inhibition of E-cadherin expression (Valastyan 2011). Although many *in vitro* supporting data exist, the exact role of the EMT *in vivo* remains to be elucidated (Tarin 2005).

Next, cancer cells must degrade the basal membrane, invade the stroma and reach blood vessels. Cancer cells cross the BM using invasive protrusions that degrade the BM components through the activity of membrane-bound metalloproteinases or the release of other specific metalloproteinases. These protrusions or “invadopodia” are also involved in directional migration and chemotaxis (Bravo-Cordero 2012). It is currently well documented that the invasive phenotype is not acquired in a cell-autonomous manner but requires interactions between cancer and host cells (Valastyan 2011). Among host stromal cells, several types are involved in tumor progression, comprising endothelial cells, pericytes and

fibroblasts. Normal fibroblasts converted into cancer-associated fibroblasts (CAF) constitute a major component of the tumor microenvironment. Fibroblasts present in benign lesions are known to suppress tumor growth, whereas the presence of CAFs supports cancer progression via the release of growth factors and proteases that remodel the ECM (Tripathi 2012). For example, Orimo *et al.* showed that CAFs residing in human breast tumors secrete high levels of CXCL12, which in turn promotes tumor growth and invasion (Orimo 2005). During invasion, the stroma is chronically inflamed. Primary tumors recruit inflammatory cells such as macrophages, through the production of chemokines (e.g., CCL2), which will increase tumor cell migration, invasion and intravasation (Pahler 2008, Joyce 2009). Tumor-associated bone marrow-derived cells, including macrophages, were shown to be the major cell type that produces proteases (Joyce 2009). These enzymes include matrix metalloproteinases, cysteine cathepsins and serine proteases. The degradation of constituents of the ECM and of cell surface proteins through the release of proteolytic enzymes facilitates the migration of cancer cells towards blood vessels. Moreover, matrix metalloproteinases process and activate cytokines and growth factors sequestered in the ECM, thereby promoting cell migration, survival and angiogenesis (Joyce 2009).

Once in the stroma, cancer cells pursue their invasion mainly via three major pathways of cell motility. First, mesenchymal-like cancer cells, originating from the EMT program, may migrate via pseudopodia-like protrusions (lamellipodia) that anchor on specific ECM components (Perlikos 2013). Second, cancer cells may adopt a more aggressive type of motility termed “amoeboid invasion” during which cancer cells gain a spherical morphology and infiltrate the stroma through pre-existing cracks in the extracellular matrix (Perlikos 2013). Amoeboid movements involve F-actin-rich protrusions (pseudopodia) and do not involve the action of proteases (Bravo-Cordero 2012). Last, cancer cells may invade the stroma collectively. Collective invasion leads to the formation of finger-like fronts in the primary tumor that intravasate and disseminate as cell clusters in the blood stream. Cell clusters can efficiently embolize blood vessels at distant sites, and have high survival rates compared to single circulating cells (Yilmaz 2010, Perlikos 2013).

1.2.2. Intravasation

Observations in renal cancer patients evidenced that tumors may shed millions of cells into the circulation every day (Glaves 1988). During early cancer progression, tumor cells proliferate, sustained by nutrients provided by host vessels. However, beyond a size of 1 mm³, the passive diffusion of nutrients and oxygen becomes limiting (the oxygen diffusion

limit through sheets of oxidative cells is $\sim 100\ \mu\text{m}$), forcing the tumor to enter a “dormancy” phase (Naumov 2006). To reinitiate growth, tumors often become angiogenic and create their own vascular network by triggering the sprouting of new vessels from preexisting ones (Carmeliet 2000, Naumov 2006). Tumor angiogenesis, primarily initiated by hypoxic conditions, is characterized by a net imbalance between pro- and anti- angiogenic factors, and leads to the formation of a chaotic vascular network. Activators of the angiogenic switch include mainly tyrosine kinase ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), whereas inhibitors include molecules such as thrombospondin-1, angiostatin and endostatin. Many proangiogenic factors are sequestered within the ECM and are released as soluble factors upon the action of matrix metalloproteinases. Similarly, many “statins” are found within the ECM as inactive precursors and are also produced via the action of proteases. For instance, endostatin is a fragment of collagen that binds to integrins, and angiostatin derives from plasminogen (Bergers 2003). Because anti-angiogenic factors also promote vascular maturation but are perpetually outcompeted by pro-angiogenic factors in growing tumors, newly formed tumor vessels remain largely immature, often lack pericyte coverage, and may have fenestrations (Tredan 2007, Geiger 2009). This leaky tumor vasculature is a facilitating factor for the systemic dissemination of cancer cells (Valastyan 2011). In addition to physical features of primary tumors, cytokines produced by cancer and stromal cells also play a pivotal role in the intravasation process. Transforming growth factor-beta ($\text{TGF-}\beta$) signaling, which is also a key modulator of the EMT program, was shown using intravital microscopy to promote single cell motility and intravasation in a breast cancer model (Giampieri 2009, Miyazono 2012). Paracrine loops between cancer and host cells recruited in the permissive tumor microenvironment improve the entry of cancer cells into the blood circulation. Wyckoff *et al.* showed that in response to the stimulation of tumor-associated macrophages (TAM) by colony-stimulating factor-1 (CSF-1) secreted by breast cancer cells, TAMs produce in turn epidermal growth factor (EGF). EGF was shown to enhance the intravasation of cancer cells (Wyckoff 2004, Wyckoff 2007). Intravasation is achieved either by production of protrusions through gaps of the endothelial wall or by passively entering the circulation through fenestrations (Perlikos 2013). It was shown that the number of intravasated cells correlated with the number of lung metastases in an orthotopic breast tumor rat model, suggesting that entry into the blood circulation may be a critical step in the metastatic cascade (Wyckoff 2000).

1.2.3. Life in transit and arrest at a distant site

Once into the blood stream, circulating tumor cells (CTC) are detached, exposed to immunosurveillance and shear stress generated by the blood flow. A vast majority of cancer cells quickly die in the blood stream owing to these harsh conditions (Perlikos 2013). However, a subset of CTCs develops resistance mechanisms. Some CTCs can induce an hypercoagulable state in the host organism and form large emboli with blood platelets (Gupta 2006). Elevated platelet numbers and coagulation are associated with poor prognosis in several types of cancer such as endometrial carcinoma, cervical cancer, ovarian cancer, gastric cancer, and esophageal cancer (Erpenbeck 2010). Platelets act as shields that protect cancer cells from mechanical forces. In addition, platelet-derived transforming growth factor- β secreted by platelets was shown *in vitro* to impair NK cell antitumor reactivity (Kopp 2009, Erpenbeck 2010, Labelle 2012).

The survival of CTCs depends on the residence time in the blood circulation. CTCs (20-30 μm) are quickly trapped in the first capillary bed they encounter (luminal diameter of capillaries $\sim 8 \mu\text{m}$). For instance, large colorectal carcinoma cells may be directly trapped in the liver because the portal vein drains the mesenteric circulation to the liver (Gupta 2006). It is therefore possible that some CTCs escape anoikis owing to a brief residence time in the bloodstream (Valastyan 2011). Luzzi *et al.* showed using videomicroscopy that more than 80% of B16F1 melanoma cells survived for three days post intraportal injection in the liver microcirculation (Luzzi 1998). This initial trapping is a passive mechanism and does not always reflect the final location of CTCs. It was indeed shown that CTCs can arrest in blood vessels much larger than capillaries (Al-Mehdi 2000). These observations indicate that active mechanisms involving adhesion molecules such as selectins, integrins and metadherins, can also contribute to the initial arrest of CTCs to distant vascular beds (Labelle 2012). In addition of CTCs found in the peripheral blood, disseminated tumor cells (DTC) are also found in the bone marrow of cancer patients. The bone marrow containing DTCs may constitute an additional reservoir of cancer cells that will recirculate to distant organs (e.g., colon cancer) or may become a favorable metastatic niche (e.g., breast and prostate cancer) (Pantel 2008).

1.2.4. Extravasation

Once entrapped in a capillary bed, cancer cells may directly form a colony in the blood lumen. The resulting micrometastasis eventually ruptures the vessel wall, and cancer

cells are allowed to invade the adjacent parenchyma. This process was shown to occur in a mouse model of lung metastases (Wong 2002). Alternatively, cancer cells may cross the layers formed by pericytes and endothelial cells to reach the stroma. This process, known as extravasation, generally occurs within 1 to 3 days after the release of cancer cells in the circulation (Labelle 2012). The extravasation process is similar to the diapedesis of leukocytes during inflammation, and is affected by the structure of the vessel wall (Perlikos 2013). The blood brain barrier is for instance much more impermeable to cells than the fenestrated sinusoids present in the liver or in the bones (Nguyen 2009).

As aforementioned, CTCs are often surrounded by platelets. Platelets were shown to be more than protective shields: they also enhance the extravasation process through platelet-tumor interactions (Labelle 2012). These interactions are mediated through cell-cell interactions or by the production of platelet-derived TGF- β (Labelle 2011). As a result, platelet-CTCs microthrombi release proinflammatory chemokines (such as CCL2) that recruit monocytes. Additionally, tumor cells produce tissue factor, which promotes the coagulation and attracts macrophages (Gil-Bernabe 2012). In the end, cancer cells are accompanied by different inflammatory cell types that actively participate to the extravasation process. For instance, Qian *et al.* showed that monocytes recruited at pulmonary metastatic sites can differentiate *in situ* into metastasis-associated macrophages that facilitate the extravasation of cancer cells. One of the responsible mechanisms was attributed to an increase in vascular permeability due to the secretion of VEGF by macrophages (Qian 2011).

1.2.5. Micrometastasis formation

After extravasation, cancer cells land in a foreign microenvironment different from the site of the primary tumor formation. Differences include the type of stromal cells, immunosurveillance, tissue architecture, ECM composition and the availability of growth factors and cytokines. Hence, cancer cells are not initially adapted to this foreign microenvironment (Valastyan 2011). It was shown that <3 % of intravenously injected cancer cells survived to form micrometastases (Luzzi 1998). A recent paradigm, known as the “pre-metastatic niche”, proposes that the primary tumor secretes systemic factors that prepare “the soil” for the arrival of metastatic cancer cells. Some examples of prepared niches can be found in physiological situations such as the implantation of a fertilized ovum in the uterine wall. This process is systemic and involves the recruitment of haematopoietic cells from the bone marrow to the future metastatic site. This concept might be of importance to explain the tropism of tumor cells for some tissues (Kaplan 2005, Psaila 2009).

Cancer cells have three distinct fates after their entry in the new microenvironment. First, a vast majority of extravasated cancer cells quickly dies. Second, the majority of surviving cells remain dormant. Third, a small subset of cancer cells survive, escape dormancy, and start to proliferate (Perlikos 2013). Tumor dormancy corresponds in clinical situations to the minimal residual disease (MRD) phase. MRD is a clinical situation wherein tumor cells are still detected in the blood or in the bone marrow after the resection of a primary tumor, whereas no macroscopic lesion is detectable (Hedley 2009). Tumor dormancy refers to two distinct phenomena. Cancer cells may remain quiescent at the G₀/G₁ phase for extended periods of time (cellular dormancy). Alternatively, cancer cells may proliferate and die by apoptosis at equal rates. The latter mechanism is likely to be due to a lack of angiogenesis at the micrometastasis site (angiogenic dormancy). Cellular quiescence is partly attributed to a lack of crosstalk between cancer cells and the microenvironment and to the loss of important surface receptors. A loss in appropriate growth factors in the foreign microenvironment combined with defects in tissue architecture leads to the perturbation of important signaling cascades such as integrins and EGFR pathways (Aguirre-Ghiso 2007). For what pertains to angiogenic dormancy, one has to remember that the angiogenic switch is an early prerequisite step to sustain the growth of primary tumors. It is triggered when there is a net advantage of pro-angiogenic factors over anti-angiogenic ones. When metastases occur, cancer has become a systemic disease. Hence, O'Reilly *et al.* showed that the primary tumor can release anti-angiogenic factors such as angiostatin and endostatin in the blood circulation, thus impairing the growth of distant micrometastases (O'Reilly 1996, O'Reilly 1997). The antiangiogenic role of primary tumors is attributed to the longer circulatory half-lives of anti-angiogenic factors compared to pro-angiogenic ones (Ramanujan 2000).

1.2.6. Metastatic colonization

Sometimes, cancer cells at secondary sites exit the dormancy state and start to proliferate. In the clinics, this event corresponds to relapse and affects for instance 20-45 % of all prostate and breast cancer patients (Aguirre-Ghiso 2007). At this point, it must be emphasized that only a very small subset of cancer cells survived until this step. It is consensually agreed that less than 0.01-0.02 % of CTCs are able to form macrometastases (Fidler 1970, Luzzi 1998). The underlying mechanisms explaining cancer relapse are still not clear but involve self-renewal mechanisms as well as microenvironmental modifications. In this regard, interactions between a primary tumor and its colonies may participate in the conversion of foreign microenvironments into hospitable niches (Valastyan 2011). McAllister

et al. showed that the growth of metastatic tumors was in some cases dependent on systemic signals, such as osteopontin, released by the primary tumor. Osteopontin can indeed recruit bone marrow-derived cells that support the proliferation of otherwise indolent tumor cells (McAllister 2008). Gene products that facilitate the metastatic colonization are organ-specific. In other words, molecular programs involved in metastasis formation depend on the metastatic site. For instance, interleukin-11 (IL-11) was shown to alter the signaling between osteoblasts and osteoclasts in bones. As a result, interleukin-11 enhances the formation of osteolytic bone metastases in breast cancer patients (Kang 2003). Although IL-11 is involved in bone metastases formation by breast cancer cells, different organ-specific metastasis genes are involved in lung or brain breast cancer metastases (Sethi 2011). Molecular programs involved in metastatic colonization at one site (e.g., the bone) also depend on the cancer cell type. Breast cancer bone metastases are osteolytic lesions: osteoclastic activity is increased and results in bone resorption. In contrast, prostate cancer forms osteoblastic lesions with disorganized bone deposition (Joyce 2009, Valastyan 2011).

I.3. Modeling metastasis

Many aspects of the metastatic cascade such as invasion mechanisms, the localization of secondary lesions and even the general temporal evolution of the process are highly heterogeneous depending on the cancer type. Consequently, preclinical models must be carefully selected for a proper understanding of tumor progression and dissemination. Two strategies currently exist for mouse studies: genetically engineered models of cancer (GEM), and transplantable tumor model systems (Figure 2). GEM refers to the introduction of oncogenic mutations in specific tissues. These models encompass the events from tumor initiation to early steps of dissemination (Bos 2010). As an example, *MMTV-PyMT X MMTV-iCre/CAG-CAC-EGFP* is a transgenic mouse model of mammary tumors in which cancer cells are naturally fluorescent (Ahmed 2002). This model was used to investigate, using multiphoton microscopy, paracrine interactions between tumor cells and macrophages (Wyckoff 2004). Nevertheless, these models often metastasize only to lungs and/or to lymph nodes and do not necessarily reflect the metastasis patterns occurring in clinical situations. On the other hand, grafting syngeneic or human cancer cells in immunocompetent or immunodeficient mice is the method of choice to study metastatic dissemination and colonization in relevant organs. Xenograft models mimic clinical situations in murine studies despite the lack of competent immune system, whereas syngeneic models accurately reflect

the tumor-microenvironment interface. Among transplantable tumor models, one can distinguish experimental from spontaneous metastasis ones. Experimental metastasis refers to the direct delivery of tumor cells into the blood stream. Tail vein injection is the most common injection route and results in the arrest of cancer cells in the lung capillaries. Tail vein inoculation is used to assess survival in the blood stream, vascular entrapment, lung extravasation and metastatic colonization. On the other hand, intracardiac delivery allows cancer cells to lodge in capillary beds of multiple organs, and is used to study organ-specific interactions. Finally, spontaneous metastasis refers to the orthotopic or ectopic injection of tumor cells that will first develop a primary tumor endowed with metastatic activities. Such model encompasses all the steps of the metastatic cascade (Wyckoff 2000, Fidler 2006, Bos 2010).

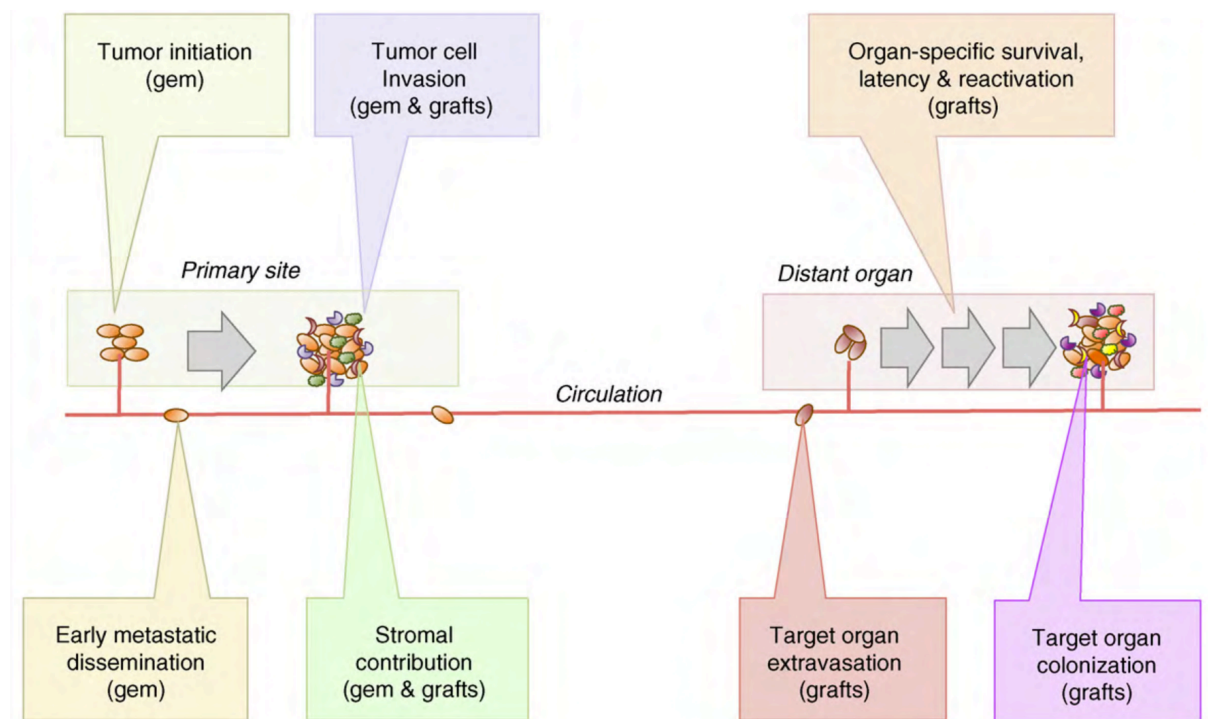


Figure 2: Use of genetically engineered models of cancer (GEM) or transplantable tumor models (grafts) for metastasis research. The use of GEMs or spontaneous models of metastasis encompasses all the steps of the metastatic cascade, whereas experimental models provide information on circulation, extravasation, homing and colonization of distant organs (Bos 2010).

II. Noninvasive cell tracking

II.1. Definition

According to Bulte *et al.*, cell tracking or cellular imaging can be defined as the “non-invasive and repetitive imaging of targeted cells and cellular processes in living organisms” (Bulte 2004). Two labeling approaches exist for tracking cells: one is direct and the other is indirect (Hong 2010, Kircher 2011) (Figure 3). Direct cell labeling consists in labeling cells of interest with contrast agents (CA). Iron oxides for MRI, ^{111}In -oxine for single photon emission-computed tomography (SPECT), 18-fluorodeoxyglucose (^{18}F -FDG) for positron emission tomography (PET) and quantum dots for optical imaging are non-exhaustive examples of CAs used in direct cell labeling strategies. Three direct labeling methods exist: *in situ*, *in vivo* and *ex vivo* (Hoehn 2007). *In situ* labeling consists in injecting the CA directly in a specific tissue for monitoring cells initially present in this area. For example, the migration of neural progenitor cells to the olfactory bulb through the rostral migratory stream was monitored using MRI following stereotactic injection of CAs (e.g., micro-sized iron oxides) into the subventricular zone (Granot 2011). *In vivo* labeling is a similar approach but requires the intravenous injection of the CA. Inflammation imaging using intravenous injections of perfluorocarbons (PFC) can illustrate this kind of application. Classical ^{18}F -FDG PET scans, considered as a molecular imaging technique, are used for the detection of glycolytic cells such as cancer cells. On the other hand, ^{18}F -FDG PET scans can be considered as an *in vivo* labeling strategy. Therefore, cellular imaging is closely related to the important field of molecular imaging (Bulte 2004). Molecular imaging is defined by Weissleder *et al.* as “the *in vivo* characterization and measurement of biologic processes at the cellular and molecular level” (Weissleder 2001). After *ex vivo* labeling with a CA, cells are injected in a living organism and are detected by appropriate imaging protocols. This latter strategy is suitable for monitoring a defined cell population because the exact nature of the labeled cells is obviously known prior to the experiment.

All direct cell labeling approaches suffer from three main limitations. First, the intracellular content of CAs is diluted with cell proliferation. Walczak *et al.* imaged neural stem cells labeled with iron oxides injected into the lateral ventricle of the mouse brain and observed a pronounced mismatch between MRI data and the histologically determined cell distribution. Moreover, this dilution effect was accentuated owing to asymmetric division of labeled cells (Walczak 2007). Second, intracellular labels can be transferred to neighboring

cell (exocytosis or cell death). As a consequence, the observation period is generally short using direct cell labeling approaches and depends on the survival and on the proliferation rate of the labeled cells. Last, direct cell tracking strategies give information on the localization of the labeled cells, but not on their function/viability (Xu 2011).

On the other hand, indirect cell labeling approaches rely on the use of reporter genes, i.e., engineered genetic constructs allowing the visualization of cells or molecular events. Reporter genes are generally expressed only by live cells, and the reporter gene is preserved in daughter cells after division. If cells stably express the reporter gene, cellular imaging is possible for long periods of time (Kircher 2011, Gu 2012). In addition of cellular imaging, reporter gene-based methods can be used for studying expression, trafficking and interactions of proteins (Vandsburger 2012). Depending on the promoter, the expression of reporter genes can be constitutive or inducible, limiting their potential toxicity (Alfke 2003). Several categories of reporter genes exist, and each of them has advantages and disadvantages. Receptor or transporter-based reporters require complex processes of protein trafficking and membrane integration that may affect reporter readout and quantification. Enzyme-based reporters can trap substrates carrying imaging probes or continuously produce signals through reaction catalysis. Enzyme-based reporters are thus expected to produce strong signals. Finally, intracellular reporter systems require that the substrate can access the reporter protein, whereas the substrate can remain in the extracellular compartment for receptor or transporter-based systems (Brader 2013). Luciferase for bioluminescence imaging, thymidine kinase for PET/SPECT and ferritin for MRI are some examples of reporter genes used in cell tracking studies.

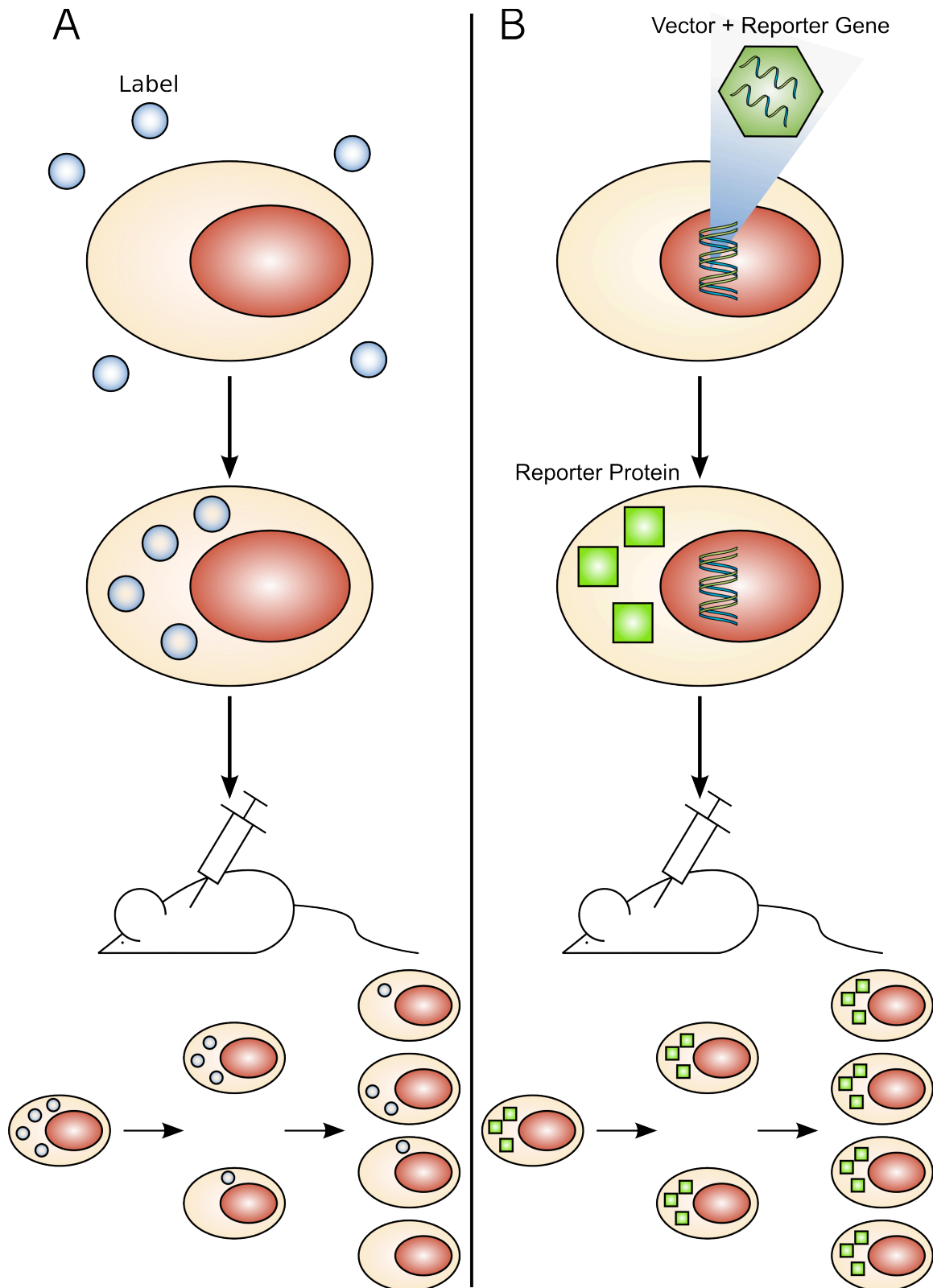


Figure 3: Principles of *ex vivo* direct and indirect labeling methods for cell tracking. (A) For *ex vivo* direct labeling experiments, cells of interest such as macrophages, stem cells or cancer cells are incubated with a contrast agent before their injection in a small animal. Following injection, imaging the labeled cells is possible for a limited period of time owing to the dilution of the signal with cell proliferation. (B) In indirect cell labeling approaches, cells are transfected (or transduced using lentiviral vectors) with reporter genes incorporated in a vector containing a promoter. The promoter can be either constitutive or inducible: it regulates the expression of the reporter gene. If stably expressed, the reporter gene is passed on to daughter cells, allowing long-term imaging of the cells of interest *in vivo* (adapted from Kircher 2011).

II.2. Goals of cell tracking

The ability of monitoring the fate of cells is mandatory for the development of cell-based therapies and in fundamental cancer research (Kircher 2011). Cell-based therapies consist in delivering living cells to cure pathologies that are resistant to conventional treatments. Stem cell transplantation is still an experimental procedure. There is an important gap between the promising preclinical studies and the limited benefits observed in clinical trials. With regard to cardiac repair after myocardial infarction, numerous clinical trials showed limited or moderate benefits of cellular therapies on the cardiac function (Wei 2010). For instance, the BOOST clinical trial assessed the effect of a single intracoronary infusion of autologous bone marrow cells in patients with myocardial infarction. Improvement of the left ventricle systolic function was observed 6 months after transplantation but was not sustained after 5 years (Meyer 2009). These rather disappointing results are mostly due to the lack of understanding of the distribution, function and survival of stem cells in their local microenvironment (Gao 2013). Currently, long-term assessment of functional responses is the only available indicator of success or failures of cellular therapies. In classical drug development, pharmacokinetics studies are used to quantify the biodistribution of the drug in a living organism. Such information is difficult to obtain in cell-based therapies (Janowski 2012). Cell tracking methods thus represent an alternative to invasive histological studies for monitoring the distribution of therapeutic cells (Gao 2013). To date, invasive methods such as intravital imaging using two-photon microscopy are still necessary for assessing cell function (Pesic 2013). Nevertheless, the expression of some genes was non-invasively imaged *in vivo* using reporter genes constructs. For instance, the chondrogenic differentiation of mesenchymal stromal cells was documented using dual luciferase labeling for BLI. Cells were transduced with a procollagen II promoter driving a firefly luciferase reporter gene and with a constitutive *renilla* luciferase gene using a cytomegalovirus promoter. Procollagen II expression was directly linked to acquisition of a chondrocytic phenotype, whereas *renilla* luciferase expression reflected cell survival (Vilalta 2009). However, the use of reporter genes requires genetic manipulations of the cells and could alter cell functions (Frangioni 2004). For monitoring cell survival, it has been shown that contrast remained present when cells labeled using a direct approach died. Therefore, reporter gene-based methods are preferred for the determination of cell survival *in vivo* (Chen 2009).

According to Frangioni et al., an ideal cell tracking method should meet the following criteria (Frangioni 2004):

1. The label should be biocompatible and non-toxic.
2. No genetic alteration to the labeled cell.
3. Single-cell detection at any anatomical location.
4. Quantification of cell number.
5. Minimal dilution of labels with cell division.
6. No transfer of the label to other cell types.
7. Non-invasive imaging in the living organism for long periods.

Unfortunately, such method currently does not exist and the choice of the imaging method depends on the application. With regard to cell-based therapies, stem cells will exert different functions depending on the pathology. The main objectives of cellular therapies are regeneration, replacement or redirection (Figure 4). In case of a damaged tissue with low regenerative potential such as the heart or the central nervous system (CNS), stem cells are injected to regenerate the wounded tissue (regenerative function). For regenerative cellular therapies, ensuring the exact localization of stem cells is mandatory. Due to its high spatial and temporal resolution, MRI has been extensively investigated for stem cell tracking in CNS pathologies such as stroke or spinal cord injury (Dousset 2008). Of note, cell viability must be verified *in vivo*. Quantification of the number of injected cells is also mandatory. Indeed, the therapeutic response is directly linked to the number of surviving therapeutic cells after injection (Aarntzen 2012). Second, cells can be transplanted to restore an appropriate exocrine function (replacement function). For instance, pancreatic islet transplantation is extensively studied to restore a physiological production of insulin in diabetic patients. For such purpose, knowing the exact cell localization is less important since the implantation site is less critical. Indeed, pancreatic islets have been transplanted in preclinical studies into various sites such as the liver, the renal subcapsule or the spleen (Merani 2008). The host site must be chosen to ensure that the cellular physiology and the exocrine function of pancreatic islets are preserved. The long-term survival, the function (i.e., insulin secretion) and the number of engrafted pancreatic islets are thus important parameters for effective replacement cellular therapy. Up to now, no single imaging method is considered as a method of choice for imaging transplanted pancreatic islets as each method suffers from important limitations, e.g., sensitivity for MRI and resolution for PET/SPECT (Andralojc 2012). Third, therapeutic cells can also be injected to modulate the function of a diseased tissue (redirection function). In such case, the transplanted cells are intended to tune the surrounding tissue via paracrine interactions. Cancer vaccination consists in delivering immune cells or stem cells to the tumor

in order to stimulate an immune response. For example, dendritic cells can be injected in lymph nodes to induce cancer-specific immune responses. Alternatively, adoptive T cell cancer therapy consists in delivering manipulated cytotoxic T cells that will selectively target and kill cancer cells. Many cell tracking studies have explored the feasibility of cancer immunotherapy in preclinical and clinical studies. For assessing redirection processes, knowing the number of cells and the tissue localization of the transplanted cells are mandatory. Because therapeutic cells might be short-lived for cancer immunotherapies, short-term (direct) cell tracking strategies are well suited for imaging cancer immunotherapies (Hong 2010, Aarntzen 2012).

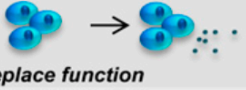
Medical condition	Role of therapeutic cells	Examples	Key points for imaging			
			Localization	Viability	Function	Cell number
Tissue damage	 <i>Regenerate tissue</i>	Stem cell transplantation in cardiovascular disease	+++	+++	++	+++
Autoimmune disease	 <i>Replace function</i>	Pancreatic islet transfer in insulin-dependent diabetes	+	+++	++	+++
Cancer	 <i>Redirect processes</i>	Dendritic cells to induce tumor-specific immune responses	+++	++	+	+++

Figure 4: The three main objectives of cellular therapies (regeneration, replacement and redirection) and key points for imaging therapeutic cells (Aarntzen 2012)

In cancer metastasis research, probing the distribution of cells is mandatory for elucidating the mechanisms of the metastatic cascade. As a secondary tumor can arise from one single cell (Fidler 1986), the cellular imaging method must be very sensitive. Currently, intravital microscopy allows single cell imaging and is the most appropriate technique for studying cell-cell interactions or even cellular processes *in vivo*. This technique played a pivotal role for assessing (i) cancer initiation and growth, (ii) the interaction between cancer cells and the stroma at the primary tumor and at metastatic sites, (iii) intra- and extravasation, (iv) responses of cancer cells to chemotactic factors, and (v) responses of cancer cells to anti-cancer treatments (Condeelis 2003, Bonapace 2012). Intravital imaging was also used for tracking the fate of cells after implantation (Figure 5). Nevertheless, cell tracking using intravital imaging is possible for a short time period (few hours) and in a confined environment ($<0.1 \text{ mm}^3$) (Pittet 2011).

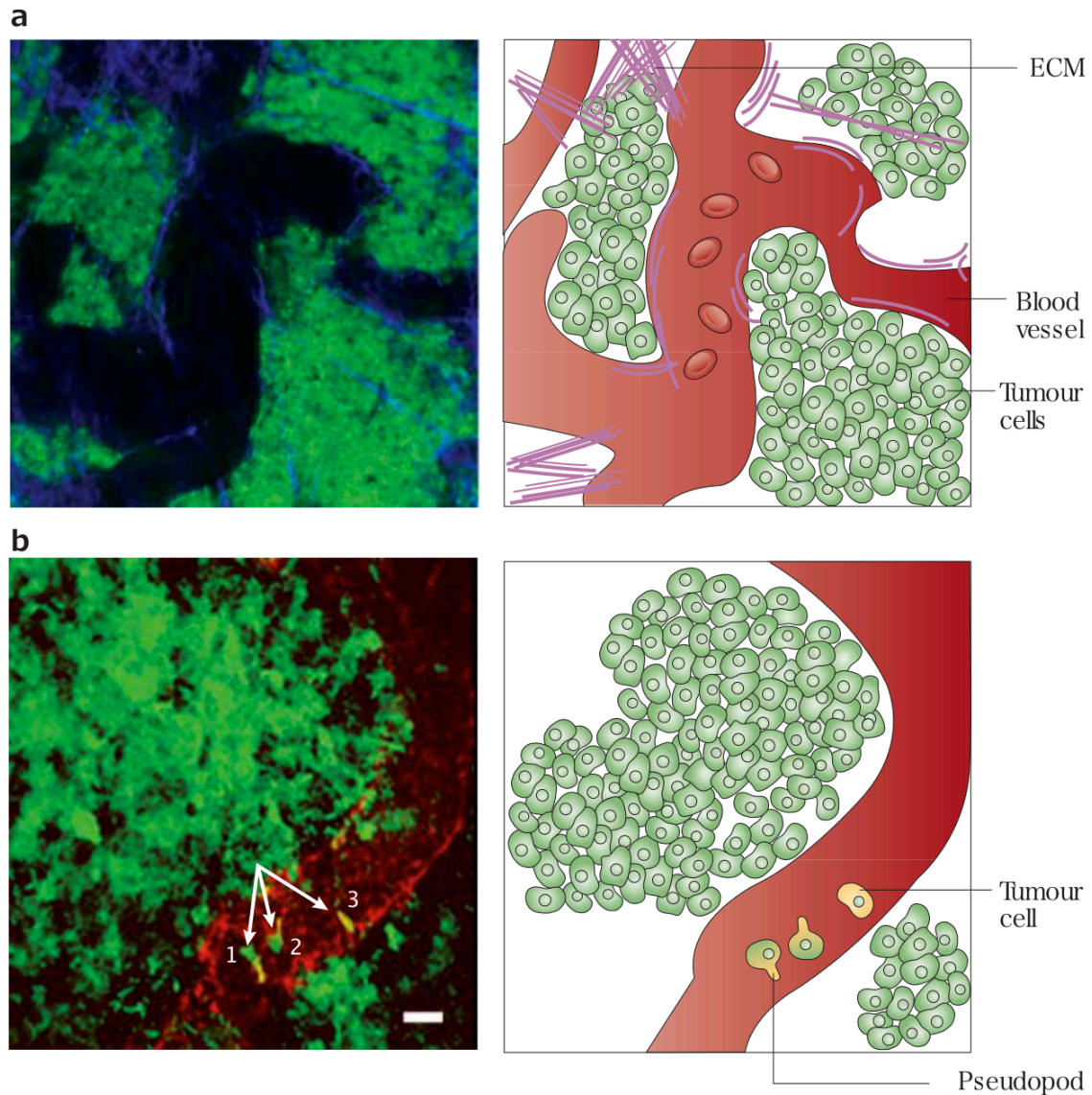


Figure 5: Use of intravital microscopy for assessing tumor cell intravasation. (A) Tumor cells labeled with green fluorescent protein (GFP) are present next to blood vessels. ECM fibres (purple) line blood vessels. (B) Intravasation of cancer cells. Blood vessels were stained in red using intravenous administration of rhodamine dextran. Arrows indicate the presence of cells that crossed the vascular wall and shed into the blood vessel (Condeelis 2003).

II.3. Whole body imaging techniques for noninvasive cell tracking studies

As summarized in Table 2, the main imaging techniques used in cell tracking studies are MRI, radionuclide imaging (PET and SPECT) and optical imaging (fluorescence and bioluminescence). MRI and PET/SPECT are routinely used in clinical practice, whereas light scattering by tissues restricts the use of optical imaging to small animal studies. The wide variety of pulse sequences allowing the use of numerous sources of contrast and the high anatomical resolution are the main advantages of MRI. On the other hand PET/SPECT imaging is often combined with computer tomography (CT) to provide anatomical details of

the localization of radionuclides. PET/SPECT can detect few molecular events *in vivo*. Moreover, quantification is possible with PET (Hicks 2012). Finally, optical imaging is particularly well suited for small animal studies. This imaging method is user-friendly and cheap compared to MRI and PET/SPECT. Short acquisition times combined with a high sensitivity allow repetitive imaging of optically labeled probes. Details about each technique will be given in the following sections (Massoud 2003, Condeelis 2010, Fu 2011).

Table 2: Overview of imaging methods of cell tracking studies in the small animal. FMT, fluorescence-mediated tomography (Massoud 2003, Condeelis 2010, Fu 2011).

Technique	Resolution	Sensitivity	Depth	Advantages
MRI	25-100 μm	10^{-3} - 10^{-5} M	No limit	• High soft tissue contrast • Numerous source of contrasts
PET	1-2 mm	10^{-11} - 10^{-12} M	No limit	• Many different tracers available
SPECT	1-2 mm	10^{-10} - 10^{-11} M	No limit	• Simultaneous imaging of different radiolabels
Fluorescence imaging	1 mm	10^{-9} - 10^{-12} M	<1 cm (2-3 cm using FMT)	• Rapid screening of surface molecular events
BLI	Several mm-cm	10^{-15} - 10^{-17} M	<3 cm	• Repetitive imaging of transgene expression <i>in vivo</i>

II.4. Magnetic resonance imaging

II.4.1. Contrast mechanisms

Once submitted to the external magnetic field generated by a MRI scanner, proton spins contained in water molecules behave like small magnets and align with the external magnetic field. The alignment of proton spins creates a longitudinal magnetization. Additionally, proton spins also precess at a frequency that is characteristic of the environment and, thus, of the external magnetic field. If a radiofrequency (RF) pulse is applied at a frequency equal to the precession frequency of protons spins, the magnetic resonance phenomenon occurs. Protons spins absorb the external energy from the RF pulse and the net longitudinal magnetization decreases. Meanwhile, proton spins start to precess in phase, creating a net oscillating transversal magnetization perpendicular to the external magnetic field. After the RF pulse, protons realign to the external magnetic field and the stored energy is released. The transversal magnetization decreases, whereas the longitudinal magnetization returns to its initial state. This phenomenon constitutes the relaxation. To characterize the recovery of the magnetization along the external field, the longitudinal relaxation time T_1 is used. The time constant T_1 is the time needed for recovering 63% of the initial longitudinal

magnetization. The loss of phase of proton spins after the RF pulse is responsible of a loss of transversal magnetization, i.e., the transversal relaxation. Without any magnetic field inhomogeneities, the signal decays along the transversal plane with a time constant called T_2 . The transversal relaxation time T_2 is the time needed for the transversal magnetization value to drop to 37% of its initial value. The transversal relaxation time T_2 is shorter than the longitudinal relaxation time T_1 and depends primarily on spin-spin interactions (Figure 6). In practice, uncompensated external magnetic field inhomogeneities shorten the transversal relaxation process. In this case, the signal decays along the transversal plane with a time constant called T_2^* . The transversal relaxation time T_2^* depends on both external magnetic field inhomogeneities and spin-spin interaction. T_2^* is always shorter than T_2 .

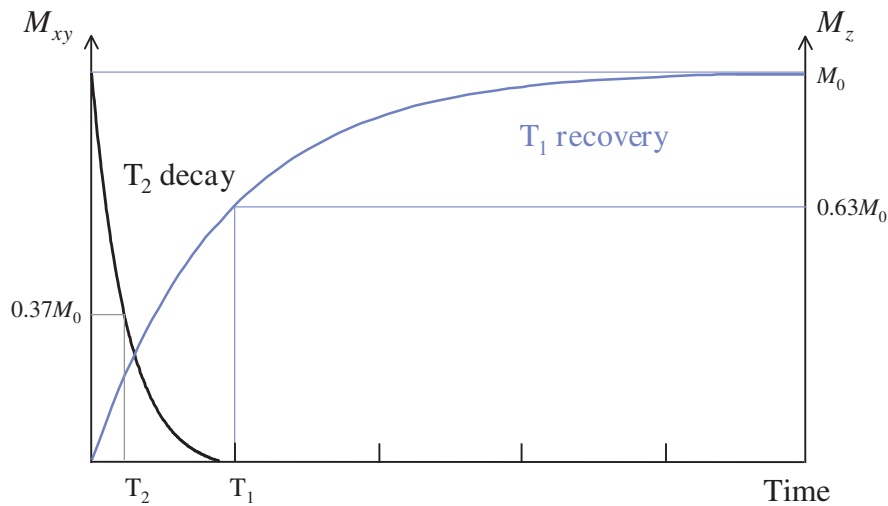


Figure 6: Longitudinal (blue) and transversal (black) relaxation after a RF pulse. T_1 and T_2 relaxation occur simultaneously but T_2 is quicker than T_1 . M_{xy} indicates the transversal magnetization, whereas M_z indicates the longitudinal magnetization (McRobbie 2006).

Contrast, i.e., the difference of signal between two regions, is based on the different relaxation properties of tissues and the concentration of protons spins. Relaxation characteristics for a given tissue mostly depend on the temperature, the external magnetic field strength and the mobility of water protons. In practice, tissues with different relaxation times can be visualized using T_1 -, T_2 - and T_2^* -weighted images. Finally, proton density images reflect the abundance of protons in a given tissue. Nevertheless, endogenous contrast is sometimes weak and the use of exogenous contrast agents (CA) is therefore mandatory to distinguish two distinct tissues. CAs shorten the relaxation times of surrounding protons (T_1 and/or T_2). The relaxivity defines the ability of a CA at a concentration of 1 mM to enhance the relaxation rate of neighbouring protons in a homogenous medium. The relationship

between the relaxivity and the relaxation rate (inverse of relaxation time) is given by the following equation:

$$R_{1,2} = R_{1,2}^0 + r_{1,2} \cdot C$$

where $R_{1,2}$ is the respective T_1 - or T_2 -proton relaxation rate ($1/T_{1,2}$; unit, s^{-1}) in the presence of the CA. $R_{1,2}^0$ is the relaxation rate in the absence of the contrast agent, and C is the contrast agent concentration (unit, mM). $r_{1,2}$ is the relaxivity (unit, $s^{-1} \text{ mM}^{-1}$) (Bjornerud 2004). This relationship is true when the CA is homogeneously dispersed in a solvent. In practice, the distribution of the CA in the voxel (i.e., “a three-dimensional pixel”), the proton density, the diffusion and the chemical environment also influence the generated contrast (Burtea 2008). Among CAs, one can distinguish:

- Paramagnetic CAs (e.g., gadolinium) having a low r_2/r_1 ratio (close to the unity) and inducing hyperintense signals (bright spots) on T_1 -weighted images. These CAs are also called “positive” contrast agents.
- Superparamagnetic CAs (e.g., iron oxides) having generally a high r_2/r_1 ratio and inducing hypointense signals (dark spots) on T_2 -weighted images. Superparamagnetic CAs are also called “negative” contrast agents.

II.4.2. Direct labeling

II.4.2.1. Paramagnetic agents

Lanthanides derivatives such as Mn^{++} ions or gadolinium chelates belong to the class of paramagnetic CAs. The presence of unpaired electrons (7 unpaired electrons for Gd^{+++}) is responsible for the paramagnetic properties of these metallic ions. Gadolinium-based CAs are the most commonly used CAs in MRI. The use of chelates prevents the toxicity of gadolinium ions. Gd-DTPA (Magnevist[®]), Gd-DOTA (Dotarem[®]), Gd-DTPA-BMA (Omniscan[®]) and Gd-HP-DO3A (Prohance[®]) are extracellular agents owing to their hydrophilic nature. These CAs are for example used for brain tumor detection. Brain tumors display blood brain barrier disruptions and extracellular agents are allowed to extravasate into these lesions where they induce hyperintense signals (Jestaedt 2012). Gd-based CAs are also used for MR angiography (Knopp 1999, Hartung 2011) or liver tumor detection (Park 2012).

Gadolinium chelates were the first paramagnetic CAs used for labeling cells. One main advantage compared to T_2 CAs is that T_1 -positive contrast is much easier to observe than T_2 negative contrast. The latter can indeed be confounded with other intrinsic contrast sources such as air cavities, hemorrhage and blood vessels (Himmelreich 2009).

Two mechanisms explain the paramagnetic relaxation. First, the “inner sphere” relies on the close interaction between water molecules and the electronic spins of the paramagnetic center. The inner sphere contribution is influenced by several factors such as the relative concentration of paramagnetic CAs and water molecules, the number of water molecules in the first coordination sphere (q), the water residence time (τ_M) and the rotational correlation time of the hydrated complex (τ_R). Second, the “outer sphere” contribution relies on the long-distance dipolar interaction between paramagnetic centers and nuclear spins (Burtea 2008). Unlike superparamagnetic CAs, an effective contrast of Gd-based agents requires a close proximity between the paramagnetic centers and water protons. After cell internalization, interactions between water molecules and Gd-based CAs are modified, resulting in a net decrease in T_1 effects (Modo 2002). Concerning sensitivity, phantom studies at 7 T showed that the detection threshold of endothelial progenitor cells labeled with gadolinium chelates (Gd-HPDO3A) was ~2000 cells (Crich 2004).

Hydrophilic chelates are not well adapted for cell labeling studies. Indeed, the cell membrane is not permeable to gadolinium chelates and about 10^8 Gd per cell (~0.1 femtomol per cell) are needed to produce positive contrast (Aime 2002, Faucher 2012). Therefore, high concentrations and prolonged incubation times are needed for passive labeling with such CAs. After internalization, gadolinium compounds might be dechelated and release free, toxic Gd^{3+} ions into the cell. Cationic liposomes or microemulsions containing high levels of amphiphilic paramagnetic moieties have been used to improve the labeling efficiency (Liu 2009). Another main limitation of gadolinium compounds is the lack of sensitivity. Sensitivity can be improved either by increasing the amount of gadolinium atoms per particle or by optimizing the longitudinal relaxivity of Gd-based CAs (Strijkers 2007). Grafting lanthanide chelates to polymeric scaffolds such as dendrimers or dextrans results in high loads of paramagnetic centers (Strijkers 2007, Liu 2009). Other gadolinium agents have been proposed such as carbon cages made of nanotubes or metallofullerenes. Although a study showed that mesenchymal stem cells labeled with gadolinium fulleranol had 10-times higher relaxivities than cell labeled with gadolinium chelates, a significant decrease in cell proliferation was observed (Anderson 2006, Liu 2009). Tran *et al.* labeled mesenchymal stem cells using short (20-80 nm) segments of single-walled carbon nanotubes encapsulating small clusters of Gd^{3+} . Transmission electron microscopy confirmed the intracellular localization of these nanotubes

after labelling, and MRI performed on 10^7 labeled mesenchymal stem cells demonstrated a nearly two-fold reduction in T_1 relaxation time compared to unlabeled cells at 1.5 T. Of note, the labeling procedure did not exert cytotoxicity (Tran 2010). Recently, the need of effective paramagnetic CAs for cell labeling purposes has led to the development of gadolinium oxide (Gd_2O_3) nanoparticles. Among all Gd-based contrast agents, these particles have the highest gadolinium atom density (200-400 Gd per particle). Because of their surface-volume properties, Gd_2O_3 nanoparticles have higher longitudinal relaxivities compared to gadolinium chelates. Faucher *et al.* used PEGylated ultrasmall gadolinium oxides ($r_1 = 14.2 \text{ mM}^{-1}\text{s}^{-1}$ at 1.41 T, $r_2/r_1 = 1.20$) for labeling and tracking F98 glioblastoma cells implanted in the mouse brain (Figure 7). The authors showed that Gd_2O_3 -labeled cancer cells produced a hyperintense signal on MR images at 1 T, whereas unlabeled cells did not produce any contrast. As an additional control, the same experiment was carried out using commercial iron oxides (Molday Ion Rhodamine B[®]) as labeling agent. The sensitivity was higher on T_2^* -weighted images with iron oxide-labeled cells, but anatomical information around the site of implantation was less accurate compared to T_1 -weighted images with Gd_2O_3 -labeled cells (Faucher 2012).

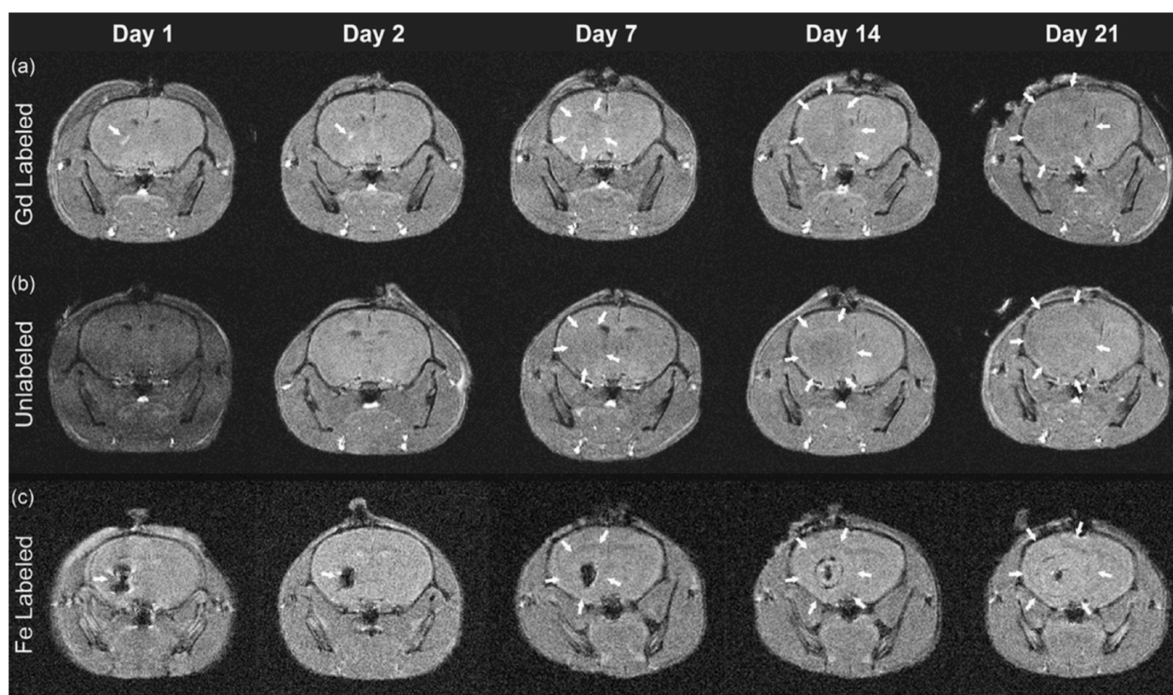


Figure 7: MRI of mouse brains injected with 3.10^5 F98 cells. (A) Gd_2O_3 -labeled and (B) unlabeled cells (T_1 -weighted images). A bright spot due to the presence of Gd_2O_3 -labeled cells is detectable on day 1 and 2. (C) A dark spot owing to the presence of 3.10^5 iron oxide-labeled cells is visible on T_2^* -weighted images. Whatever the labeling protocol is (Gd_2O_3 or iron oxides), contrast is progressively lost with time owing to tumor growth. Arrows represent implanted cells (days 1 and 2) or delineate the tumor size (days 7-21) (Faucher 2012).

Manganese is a paramagnetic transition metal that possesses 5 free electrons and produces T_1 contrast. In experimental studies, an interesting feature of Mn^{++} is its ability to mimic Ca^{++} *in vivo*. As calcium, manganese ions enter in cells via voltage-gated Ca^{++} channels. Mn^{++} can be taken up by neurons and move along axons to reach synapses (Silva 2008, Eschenko 2012). Hence, manganese-enhanced MRI (MEMRI) was increasingly developed to study the functional connectivity patterns of axons in small animals (Pautler 1998, Inoue 2011). MEMRI is mostly performed using infusions of $MnCl_2$. However, as Mn^{++} is neurotoxic when it accumulates in the striatum (Gallez 2001, Dorman 2006, Rivera-Mancia 2011, Roth 2013), gadolinium chelates were preferred as MRI contrast agents. Still, recent formulations of manganese oxide nanoparticles hold promises for cell tracking applications. For instance, mesoporous silica-coated hollow manganese oxides ($r_1=0.99 \text{ mM}^{-1} \text{ s}^{-1}$ at 11.7 T) were used for labeling and tracking adipose-derived mesenchymal stem cells in the mouse brain over 14 days at 11.7 T (Kim 2011).

II.4.2.2. ^{19}F MRI

Other nuclei than ^1H have net nuclear spin. Hence, it is also possible to image other elements such as ^{19}F , ^{31}P and ^{13}C using appropriate coils. ^{19}F MRI is based on the detection of the nuclear magnetic resonance (NMR) signal produced by fluorine atoms. NMR sensitivity of ^{19}F is about 83% of ^1H per nucleus. The lack of endogenous signal in living organisms owing to very low concentrations of mobile fluorine atoms provides to this technique an important contrast to noise ratio. Signal is only detected after administration of exogenous fluorinated probes such as perfluorocarbons (PFC) (Ruiz-Cabello 2011), i.e., hydrophobic molecules carrying multiple C-F bounds that are considered to be inert and non-toxic agents. PFCs act more like tracers compared to contrast agents used in ^1H MRI, inducing « hot spots » due to elevated local concentrations of fluorine atoms. Anatomical ^1H images performed during a same MRI examination can be surimposed to fluorine MR images to precisely localize the fluorinated tracers after administration. Another major advantage of this method is that the number of ^{19}F atoms can be quantified by integrating signals in spin density-weighted images. External ^{19}F reference standards must be added in the image field of view for absolute quantification (Ahrens 2013).

The most common PFCs used in ^{19}F MRI are perfluoropolyether (PFPE), perfluorooctylbromide (PFOB) or perfluoro-15-crown-5-ether (PFCE). One of the first cell tracking study using ^{19}F MRI aimed to visualize macrophages labeled with PFCE in the central nervous system of a rat model of allergic encephalomyelitis (Noth 1997). The

formulation, the nature of PFCs and the phenotype of the cell must be taken into account for labeling cells. PFCs are insoluble in water and are often used as microemulsions for cell labeling. The most common formulation consists in a perfluorocarbon coated with a lipid surfactant (e.g., phospholipids or poloxamers). Fluorescent dyes (such as rhodamine) and targeting moieties can also be added. Phagocytic cells easily take up PFCs, whereas additional transfection agents, cationic lipids or antibodies may be needed to enhance fluorine labeling of non-phagocytic cells (Srinivas 2012). A cell tracking study using PFCE-labeled neural stem cells evidenced that cationic emulsions showed a better uptake than anionic preparations. Neural stem cells labeled with this cationic fluorine formulation were next injected into the left striatum, and were imaged with a constant ^{19}F signal for a period of 1 week (Ruiz-Cabello 2008). To maximize the sensitivity, PFCs should carry a maximum of fluorine atoms and also have a simple ^{19}F NMR spectrum with one resonance peak. The linear PFC PFOB displays multiple peaks but only one single peak can be used for imaging. Other peaks must be suppressed prior to imaging to avoid chemical shift artifacts. On the other hand, PFCE is a macrocyclic PFC with 20 equivalent fluorine atoms and has one single NMR resonance. Finally, PFPE has one resonance peak, carries more than 40 ^{19}F atoms and has end groups amenable to chemical modifications (e.g., conjugation with a fluorescent probe) (Ahrens 2013).

Two strategies currently exist for tracking cells using ^{19}F MRI. *Ex vivo* labeling is the first approach for tracking specific cell populations after injection in living organisms. If the amount of ^{19}F atoms internalized within the cell is known and if the labeling protocols are reproducible, quantification of cells of interest is feasible *in vivo*. *Ex vivo* fluorine labeling has been successfully performed on dendritic cells, T cells and stem cells (Ahrens 2013). For instance, neural stem cells labeled with a bimodal agent carrying a fluorescent and a ^{19}F probe were implanted in a stroke-damaged rat brain. Fluorine MRI revealed the presence of labeled stem cells in the damaged area. Approximately 20 % of the label was found in host cells one day after injection, indicating that fluorine compounds were released from dead cells, and the ^{19}F signal was significantly decreased 7 days after implantation (Bible 2012). Partlow *et al.* reported that stem/progenitor cells labeled with PFOB or PFCE nanoparticles could be discriminated on MR images at 11.7 T *in vivo* owing to different fluorine signatures (spectral frequencies). Moreover, no adverse effect was observed on cell viability or function after labeling (Partlow 2007). This lack of toxicity was observed in another study involving human dendritic cells labeled with PFPE. No difference in viability and function (maturation, phenotype, cytokine production, T-cell stimulatory function and chemotaxis) was observed between PFC-labeled and unlabeled cells (Helfer 2010). *Ex vivo* PFC-based cell labeling was

used to track various cell types such as stem cells and cancer cells. In addition of the localization of labeled cells, some information on the cell functions can be extracted. Using PFCE labeling, Kadayakkara *et al.* visualized glioma cells implanted in the rat brain (Figure 8). As a linear relationship exists between the ^{19}F R_1 and the partial pressure of oxygen (pO_2) owing to interactions between fluorine atoms and paramagnetic oxygen, the intracellular oxygenation was also assessed after treatment with bischloroethylnitrosourea (BCNU). BCNU is an alkylating agent used for the treatment of gliosarcoma (Kadayakkara 2010).

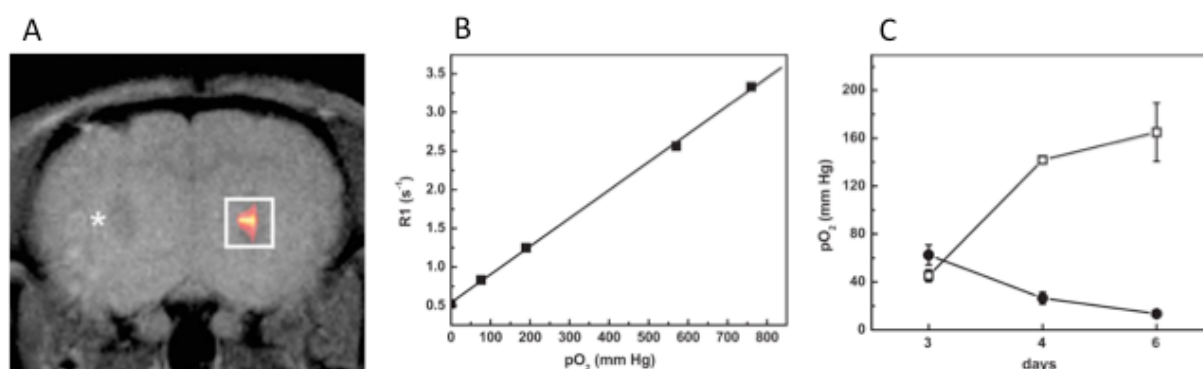


Figure 8: (A) composite $^{19}\text{F}/^1\text{H}$ image of glioma cells labeled with PFCE injected in the right striatum. (B) Calibration curve for R_1 versus pO_2 for a PFCE emulsion. (C) Evolution of the intracellular pO_2 in mice treated with BCNU ($\square$) or in control mice ($\bullet$) (Kadayakkara 2010).

In the second strategy, PFCs emulsions are directly delivered intravenously for the *in vivo* labeling of phagocytes circulating in the blood stream. Phagocytic cells are macrophages, monocytes and dendritic cells that participate in inflammatory events. The number of fluorine atoms quantified in spin density-weighted images after *in vivo* labeling is proportional to the inflammatory response (Ahrens 2013). Nevertheless, the exact phenotype of the ^{19}F -labeled cells is unknown and cell quantification is not possible. The number of ^{19}F atoms taken up by cells cannot be determined after *in vivo* labeling. This labeling strategy was exploited for studying the trafficking of phagocytic cells in several inflammatory events such as bacterial infection, cardiac and cerebral ischemia and organ transplant rejection (Ahrens 2013).

Sensitivity is however a main limitation of ^{19}F MRI. Using recent small animal imaging systems (11.7 T), the sensitivity of ^{19}F MRI is $\sim 10^{16}$ fluorine spins/voxel for an acquisition time of 1 h (Boehm-Sturm 2011). ^1H MRI is based on the detection of the NMR signal arising from water protons (two thirds of all nuclei present in a living organism), but the number of fluorine atoms introduced in cells after PFC labeling ranges from 10^{11} to 10^{13} ^{19}F /cell (Ahrens 2013). Studies showed that approximately 10^4 - 10^5 cells/voxels (clinical MRI

scanners) and 10^3 - 10^4 cells/voxel (preclinical high field MRI) can be detected using ^{19}F MRI cell tracking (Ahrens 2013).

II.4.2.3. Superparamagnetic iron oxides

The iron oxides used for imaging are made of a superparamagnetic core containing magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) coated with organic polymers such as dextran, carboxydextran, polyethylene glycol, polystyrene/divinylbenzene or silica. Coating improves the stability and the biocompatibility of the nanoparticles and prevents aggregation (Laurent 2008, Gossuin 2009, Himmelreich 2009, Cromer Berman 2011).

Iron oxides are classified depending on their size, crystalline structure and the nature of their coating (Strijkers 2007). Micron-sized iron oxides (MPIO) are the biggest particles with diameters comprised between 0.9 and 1.63 μm (Shapiro 2005). Superparamagnetic iron oxides (SPIO) are smaller particles (hydrodynamic diameter >50 nm) with several iron oxide crystals. Because they accumulate in Kupffer cells after intravenous administration, they were initially designed for liver imaging. Ferumoxides (Endorem[®]) and Ferucarbotran (Resovist[®]) were commercial SPIO used for the detection of liver tumors and metastasis (Di Marco 2007). Ultrasmall superparamagnetic iron oxides (USPIO, hydrodynamic diameter <50 nm) have a single iron oxide crystal and are characterized by a prolonged blood half-life compared to SPIO (ranging from 80 minutes to >24 h for USPIO compared to 2-4 h for SPIO). USPIO are used for MR angiography. Owing to their small size and hydrophilic coating, they are not directly recognized by phagocytic cells of the mononuclear phagocyte system. They are able to cross capillary walls, allowing them to reach the lymphatic system. Therefore USPIO are also suitable for MR lymphography (Elias 2009). For instance, USPIO Ferumoxtran-10 (Sinerem[®]) is used for the detection of metastatic lymph nodes (Islam 2009). Very small superparamagnetic iron oxide particles (VSOP) constitute a category of smaller particles (7 nm) and have a citrate coating (Millward 2013). Additionally, experimental dextran-coated superparamagnetic iron oxide nanoparticles include monocrystalline iron oxide nanoparticles (MION) and cross-linked iron oxide nanoparticles (CLIO). CLIO are nanoparticles in which the dextran is covalently cross-linked. They contain free amine groups that facilitate their conjugation with targeting agents (Tassa 2011).

Iron oxides such as Endorem[®], Resovist[®] or Sinerem[®] were used in the clinic before being used as labeling agents. Other nanoparticles used for *ex vivo* cell labeling, such as VSOP-C184, have been developed as blood pool agents for MR angiography (Wagner 2013). Therefore, these CAs are not optimized for cell tracking applications. New CAs are currently

developed for specific cell labeling purposes. Besides the choice of the type of iron oxide nanoparticle, numerous parameters (summarized in Table 3) must be addressed in order to perform a valid cell tracking study (Wang 2012b).

Table 3: Essential items to consider for MRI cell tracking studies using iron oxides (adapted from Wang 2012b).

Item	Description
Labeling agent	Physicochemical and contrast properties
Cell labeling evaluation	• Intracellular iron oxide content (pg Fe/cell) • Label localization after internalization • % labeled cells
Optimization of labeling efficiency	• Cell type (phagocytic <i>versus</i> non-phagocytic) • Cell density • Size, coating and concentration of iron oxides • Incubation time • Incubation medium
<i>In vitro</i> characterization	• Label retention • Cytotoxicity
<i>In vivo</i> imaging	• Route of injection • Imaging protocol • Limit of detection • Monitorable period • Label clearance
Outcome evaluation	• Cell function • Validation (e.g., histology)

Contrast properties

Iron oxide nanoparticles belong to the class of superparamagnetic CAs. Owing to their large magnetic moment, they will induce strong susceptibility effects on T_2 -, T_2^* -weighted images. As a consequence, the R_2 and the R_2^* of surrounding protons will be substantially increased, resulting in a signal loss on MR images (“negative” CAs). Of note, the darkening effect is much more important than the actual size of the iron oxide particles. This effect, called “blooming artifact”, makes the use of iron oxides suitable for cell tracking studies (Zhang 2004). Although iron oxides mainly influence the T_2 , T_2^* of surrounding protons, a T_1 shortening effect has also been reported (Bjornerud 2004).

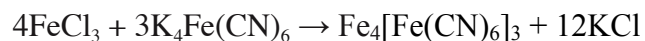
Contrast properties of iron oxides for cell labeling studies are influenced by (i) the size and the composition of the iron oxide nanoparticles, (ii) the external magnetic field strength

and (iii) the compartmentalization of iron oxides after labeling. Contrast mechanisms are dependent on the size of the particle. USPIO are characterized by a low r_2/r_1 , whereas SPIO have high r_2/r_1 . For instance, the r_2/r_1 ratio of SPIO Endorem[®] is 16, whereas the r_2/r_1 ratio of USPIO Sinerem[®] is equal to 4.5 at 1.5 T and at 37°C (Gossuin 2009). This explains why USPIO may be used as T_1 contrast agents, whereas SPIO are mainly used as T_2 -, T_2^* darkening contrast agents. The clustering of magnetic centers in large particles such as MPIO also explains their effectiveness to induce negative contrast. As a consequence, single particle imaging of MPIO was reported (Shapiro 2005). The composition and the crystallinity of nanoparticles are also critical parameters. Lee *et al.* compared the relaxivities at 1.5 T of uniform ferrimagnetic iron oxide (magnetite) nanocubes (FION) with sizes ranging from 20–120 nm to Ferumoxides and MPIO. The authors showed that the transversal relaxivity of FION ($324 \text{ s}^{-1} \text{ mM}^{-1}$) was superior to r_2 values of MPIO ($169 \text{ s}^{-1} \text{ mM}^{-1}$) and Ferumoxides ($133 \text{ s}^{-1} \text{ mM}^{-1}$) (Lee 2011). The external magnetic field also affects the r_2 of iron oxides particles. Transverse relaxivity increases with increasing magnetic field strengths until saturation. Saturation is due to the fact that iron oxides reach a maximal magnetization at a defined magnetic field strength (Bulte 1993, Gossuin 2009). The longitudinal relaxivity of iron oxides drops to zero when the external magnetic field is large enough (Gossuin 2009). During magnetic labeling, iron oxides accumulate in endosomes after cell internalization. This compartmentalization process affects both longitudinal and transverse relaxation properties. Girard *et al.* reported that r_1 and r_2 values of Ferumoxides decreased after internalization in stem cells. Interestingly, significant increases in r_2^* were also observed (Girard 2012). The drop in r_2 values after internalization was observed in another study using Ferumoxtran-10 for labeling human monocytes (Simon 2006). The size of the labeled cell and the cell type also account for modifications of the transverse relaxivities of internalized iron oxides (Rad 2007).

Cell labeling evaluation

An efficient cell tracking study requires that high yields of CAs are incorporated within cells. *In vivo* MR signal is indeed related to the amount of internalized iron oxides, although R_2 values of SPIO-labeled cells were shown to reach a plateau as a function of the cellular iron content (Boutry 2008). In most studies, iron oxide content after *ex vivo* labeling is reported in picograms of iron per cell. The iron cellular loading after incubation with iron oxide nanoparticles usually ranges from 10 to 20 pg Fe/cell (Bhirde 2011). Perls' Prussian blue colorimetry or inductively coupled plasma (ICP) spectroscopy are routinely used for iron

oxide content quantification (Boutry 2009). Perls' Prussian blue reaction catalyzes the transformation of ferric ions in the presence of potassium ferrocyanide into ferric ferrocyanide, also known as Prussian blue complex. The reaction occurs in acidic conditions:



The reaction requires complete dissolution of the iron oxide nanoparticles contained in the biological sample through acidic digestion. On the other hand, ICP spectroscopy is extremely sensitive (limit of detection: <1ppm) but is less affordable (Boutry 2009). Knowing the percentage of iron oxide-labeled cells and assessing the intracellular localization of iron oxide particles after incubation are also crucial for validating a cell labeling strategy (Wang 2012b). If iron oxides particles are merely adsorbed to cells surface, some particles are more likely to be released during *in vitro* handling and after *in vivo* transplantation (Wang 2008). Prussian blue staining or fluorescence imaging (presence of fluorochromes at the surface of the nanoparticles) is generally used for the validation/quantification of effective particle uptake.

Optimization of labeling efficiency

Cell uptake of iron oxide particles depends on the cell type, the incubation conditions, the size and the coating of the particles. Some specialized cells, such as macrophages neutrophils, monocytes and dendritic cells, are able to engulf large particles through phagocytosis. Other non-phagocytic cells, such as fibroblasts, internalize iron oxide nanoparticles through pinocytosis (fluid-phase endocytosis) (Rogers 2006, Laurent 2009). The latter can be subdivided into four categories, clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and clathrin-independent and caveolae-independent pathways (Frohlich 2012, Treuel 2013).. In general, phagocytic cells more easily take up iron oxide nanoparticles than non-phagocytic cells: passive labeling (i.e., adding particles in the culture medium) is often sufficient to label them. Increasing the iron oxide concentration or increasing the incubation time generally leads to higher yields of iron oxides internalization. In general, phagocytosis is more intense for larger particles carrying charges (Kießling 2008, Bulte 2009). Metz *et al.* showed for instance that iron oxide uptake by human monocytes was higher with SPIO Ferumoxides compared with USPIO Ferumoxtran-10. For a same class of particles, iron oxide uptake was higher with SPIO Ferucarbotran compared to Ferumoxides (Metz 2004). Ferucarbotran nanoparticles are coated with carboxydextran, a derivative of

dextran carrying negative charges due to carboxylate groups, whereas Ferumoxides SPIO are coated with neutral dextran (Mailander 2008).

If cells do not spontaneously incorporate iron oxides after simple incubation (passive labeling), several strategies have been designed to improve internalization. Cationic transfection agents, such as Poly-*L*-lysine (PLL), protamine sulfate and other molecules (such as Lipofectamine[®], Superfect[®], FuGENE[®]) have been used to facilitate the entry of iron oxides into cells (Arbab 2003a, Arbab 2004, Janic 2009). These agents, classically used for transfection, form complexes with negatively charged iron oxides nanoparticles through electrostatic interactions. The complexes, with net cationic charge, bind to anionic cell membranes and induce membrane bendings and invaginations. This process leads finally to the non-specific endocytosis of iron oxides. This approach can be applied to a wide range of cell lines (Bulte 2009, Bhirde 2011). For instance, the use of Ferumoxides-PLL complexes has been successfully applied for labeling mesenchymal stem cells, cancer cells and lymphocytes. Accordingly, mesenchymal stem cells were labeled with 16 pg Fe/cell after an incubation period of 2 h, whereas Hela cancer cells and mouse lymphocytes contained 6 pg Fe/cell and 2 pg Fe/cell, respectively, after an incubation period of 24 h. These observations also illustrate that, unrespectively of the labeling protocol, iron oxide uptake also strongly depends on the cell type (Frank 2003). As a caveat, transfection agents have been described to exert cytotoxic effects on sensitive cell lines (Arbab 2004). In addition, the benefits of combining Ferumoxides with the commonly used transfection agent lipofectamine[®] were questioned in a recent study (Soenen 2012). It was found that increasing the amount of lipofectamine[®] led to the aggregation of SPIO at the cell surface. At later time points, these aggregates were shown to be internalized in lysosomes, affecting their structure. Significant cell death was also observed (Soenen 2012). Paradoxically, anionic coatings constitute an alternative and have been shown to improve the yield of internalization of iron oxides. For instance, more efficient iron oxide uptake was achieved in 3T6 fibroblasts after labeling with Ferucarbotran compared to Ferumoxides (Boutry 2008). Such observation is in line with the study led by Mailander *et al.* who proposed the use of carboxylated SPIO (Ferucarbotran) as an alternative to the use of Ferumoxides with transfection agents (Mailander 2008). Wilhelm *et al.* further demonstrated that anionic particles chelated with meso-2, 3-dimercaptosuccinic acid (DMSA) had a more pronounced affinity for cell membranes than dextran particles (Wilhelm 2003). Although cell membranes possess negatively charged domains, anionic coatings are thought to induce fluid-phase endocytosis via binding to cationic sites of the cell membrane (Wilhelm 2003). It was also proposed that electrostatic interactions between anionic particles and the anionic cell membrane induced membrane disruptions and

subsequent internalization of the particles (Boutry 2008). The use of magnetodendrimers, i.e., iron oxides coated with a regular and highly branched anionic (carboxylate groups) or cationic (polyamine) structure, was shown to be effective for labeling cells. The charged nature of dendrimers was shown to induce endocytosis via electrostatic interactions with the plasma membrane (Bulte 2001, Rogers 2006). With regard to the influence of the size of the nanoparticles on the labeling efficiency of non-phagocytic cells, SPIO are considered as better labeling agents than USPIO (Sun 2005, Oude Engberink 2007). Large particles like MPIO contain much more superparamagnetic cores for a given particle volume compared to (U)SPIO. For instance, 1.63 μm -MPIO contain 1.1 pg Fe/particle, corresponding approximately to 1.5×10^6 SPIO or 4.3×10^6 USPIO. As a consequence, efficient intracellular iron loading can be achieved in adherent cells (75-100 pg Fe/cell) using MPIO. (Shapiro 2005, Shapiro 2008). In a systematic study, T lymphocytes were labeled with iron oxide particles with hydrodynamic diameters ranging from 33 nm (USPIO) to 1.5 μm (MPIO). Efficient labeling of cells was observed for particles up to 300 nm. However, the uptake of larger particles (MPIO) was limited (Thorek 2008). Authors highlighted the fact that MPIO suffered from gravitational sedimentation, which could hamper the magnetic labeling of non-adherent cells (Thorek 2008).

Electroporation and ultrasound pulses were also used to induce transient changes in cell membrane permeability and to enhance particle uptake by cells. Avoiding prolonged incubation times with particles in sensitive cell lines constitutes a main advantage of electro-/sonoporation. Nevertheless, these approaches can also induce significant cell death (Cromer Berman 2011). The internalization process can be further optimized by grafting the HIV-derived tat peptide on iron oxides. This peptide enables the virus to cross the plasma membrane and, therefore, to penetrate into cells (Matuszewski 2005). Lewin *et al.* efficiently labeled CD34⁺ progenitor cells with SPIO derivatized with the tat peptide (10-30 pg Fe/cell), and monitored their migration to the bone marrow of immunodeficient mice after intravenous administration (Lewin 2000). Exploiting receptor-mediated endocytosis pathways constitutes an alternative to improve uptake yields. For instance, conjugation of iron oxides with monoclonal antibodies recognizing the mouse transferrin receptor OX26 was used to label rat oligodendrocyte progenitor cells (Bulte 1999b). In another study, CD11-positive dendritic cells were labeled with SPIO conjugated to anti-CD11 monoclonal antibody. As a control, incubation of these cells with another SPIO conjugated to anti-CD4 antibodies resulted in poor labeling, because dendritic cells do not express the CD4 marker (Ahrens 2003). Recent developments have led to the synthesis of new iron oxide nanoparticles designed for cell tracking studies. For instance, adding a negatively charged (carboxymethyl)chitosan coating

(CMCS) was shown to improve SPIO uptake in mesenchymal stem cells. The iron content was equal to 27 pg Fe/cell after 24 h of incubation with CMCS-SPIO, whereas labeling with unmodified SPIO was almost five times less efficient using the same protocol (Shi 2009). Other modifications such as coating the particles with poly(N,N-dimethylacrylamide), aminosilane, 1-hydroxyethylidene-1.1-bisphosphonic acid and non-toxic protein transduction domain conjugation have been proposed to improve labeling efficiencies and toxicity profiles (Janowski 2012).

Large-size charged iron oxide particles are generally preferred to neutral particles for labeling cells. However, incubation conditions also influence the labeling efficiency. Increasing the iron oxide concentration and prolonging incubation times usually result in increased labeling efficiency, but, a plateau of intracellular incorporation of iron oxides is reached at high doses/long incubation times (Smirnov 2004, van Tiel 2010). The medium composition, the ionic strength, the pH, and the presence of serum are known to affect the physicochemical properties of nanoparticles (Maiorano 2010). Both negatively and positively charged nanoparticles bind to serum proteins and albumin. Proteins adsorbed onto nanoparticles will create a protein corona, the composition of which and the effect on cell uptake being dependent on medium composition and the type of nanoparticle (Frohlich 2012, Treuel 2013). The effects of the protein corona on cell uptake as well as potential toxicity are driven by the “cell vision”. This concept refers to the contact points between the cell surface components and the external part of the nanoparticle (Laurent 2012). Wilhelm *et al.* reported that the labeling efficiency of Hela cancer cells was significantly lower with bovine serum albumin-coated anionic maghemite nanoparticles (3 pg Fe/cell) compared to uncoated particles (40 pg Fe/cell) using the same incubation conditions (Wilhelm 2003). The cell density was also found to influence the uptake yield of iron oxides. Arbab *et al.* indeed observed an inversely proportional relationship between cell density and the intracellular iron oxide content in human cervical carcinoma Hela cells and human small-cell lung carcinoma cells (Arbab 2003a).

Label retention and toxicity

Once internalized, iron oxides initially accumulate in endosomes and some of them at later time points fuse with lysosomes. *In vitro* studies have shown that the dextran coating of Ferumoxides was degraded in lysosomal environments. As a consequence, the iron oxide core is exposed and can be dissolved at acidic pH in the presence of metallic chelates such as citrate (Arbab 2005). On the other hand, MPIO with an inert coating (styrene/divinylbenzene)

are not biodegradable and are therefore stable for long periods of time (many months) after cell internalization (Wu 2011). In addition to iron oxide metabolism, active exocytosis of the nanoparticles was reported (Wang 2008). For instance, iron oxide-labeled mesenchymal stem cells, endothelial cells and macrophages submitted to a starvation stress were shown to release microvesicles containing magnetic nanoparticles. Magnetic microvesicles were then taken up by unlabeled cells (Silva 2012). Once injected *in vivo*, labeled cells may eventually die and release their iron oxide content. Amsalem *et al.* imaged SPIO-labeled rat mesenchymal stem cells injected in the infarcted myocardium. Four weeks after transplantation, dark spots were visible on T₂*-weighted images. Histological studies showed that iron-positive cells were actually macrophages, whereas transplanted cell were not detected anymore (Amsalem 2007).

In older cell tracking studies, iron oxides were generally considered as safe labeling agents. Nevertheless, free labile iron is toxic as Fe³⁺ may react with reductants within the cell to produce Fe²⁺. The reduced form of the metal reacts then with oxygen, leading to the production of hydrogen peroxide. The presence of Fe²⁺ and H₂O₂ initiates the Fenton reaction responsible for the production of highly reactive hydroxyl radicals (Richardson 2009):



Theoretically, toxicity can affect the host organism and the labeled cells. However, iron oxide doses are low in cell tracking studies: the injection of 10⁸ cells labeled with iron oxides containing 10 pg Fe/cell represents only 1 mg of Fe, whereas classical doses of (U)SPIO in clinical practice are much more elevated (Bulte 2009). For example, the recommended intravenous dose of Ferumoxides/Endorem[®] is 0.56 mg/kg body weight (Gandhi 2006). After systemic injection, iron oxides are taken up by Kupffer cells, slowly biodegraded via the lysosomal pathway, and iron will be slowly incorporated into the hemoglobin of erythrocytes (Weissleder 1989, Elias 2009). Nevertheless, little is known about the effects of elevated SPIO levels at the injection site (e.g., intramyocardial injection of a large number of SPIO-labeled cells) (Bulte 2009). One study showed that Ferumoxides-labeled glial-restricted precursor cells, but not unlabeled cells, induced an influx of macrophages after implantation in the spinal cord (Lepore 2006).

Most toxicity studies focused on the possible deleterious effects of iron oxides on labeled cells. Although it was reported that labeling procedures have no effect on proliferation, viability, differentiation and migration (Ruggiero 2012), a study evidenced that labeling mesenchymal stem cells with Ferucarbotran affected the migration capacity and the colony formation ability of cells (Schafer 2009a). Kostura *et al.* further showed that PLL-

Ferumoxides labeling inhibited chondrogenesis in mesenchymal stem cell, whereas viability, adipogenesis and osteogenesis were not affected (Kostura 2004). As aforementioned, both Ferumoxides and Ferucarbotran, which were used in many cell labeling studies, were not designed for cell tracking applications. Moreover, these conflicting data about toxicity of iron oxides can be due to the wide range of labeling conditions and nanoparticle types used in these studies. Recently, a systematic evaluation of different classes of iron oxides (Ferumoxides, Ferucarbotran, magnetoliposomes and VSOP) showed that iron oxide labeling affected the structure of the cytoskeleton (Soenen 2010). A transient rise in reactive oxygen species (ROS) after internalization associated with a pronounced increased level of transferrin receptor-1 (TfR1) were observed, which suggested that ferric ions released from degraded nanoparticles alter iron metabolism in cells (Soenen 2011).

In vivo imaging of iron oxide-labeled cells

Ensuring an appropriate delivery of labeled cells is an obvious prerequisite step for a MRI cell tracking study. Using cellular MRI, Gorelik *et al.* have shown that immortalized human glial precursor labeled with iron oxides accumulated outside rat brain tissues after intracarotid injection (Gorelik 2012). After pterygopalatine artery ligation, cells were successfully delivered to the brain. In the first clinical trial using SPIO-based cell tracking, melanoma patients received ultrasound-guided intranodal injections of autologous dendritic cells labeled with Ferumoxides or ^{111}In -oxine (de Vries 2005). Cell tracking revealed that four of the eight patients who satisfied the end point criteria among the ten patients enrolled in the study were misinjected. MR scans containing the anatomical information showed that dendritic cells were accidentally injected into the surrounding muscle or in subcutaneous fat (Figure 9). This information was not available with scintigraphic images owing to poor anatomical details (de Vries 2005, Bulte 2009).

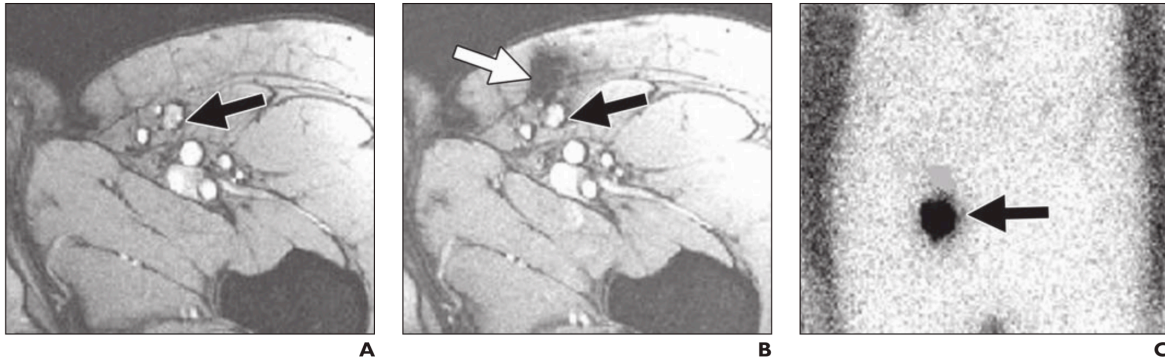


Figure 9: 35-year-old woman with stage III melanoma imaged after injection of Ferumoxides and ^{111}In -oxine-labeled dendritic cells. (A) The MR image obtained before injection shows inguinal lymph node (black arrow) as target for injection. (B) MR image obtained after injection shows the site of the accidental misinjection of labeled dendritic cells into subcutaneous fat (white arrow). Black arrow indicates the lymph node that was the intended target. (C) Scintigraphic image, because of lack of anatomic information, does not show accurate injection (black arrow) (de Vries 2005, Bulte 2009).

T_2 -, T_2^* -weighted images are used to reveal the presence of SPIO-labeled cells in cell tracking studies. As aforementioned, T_2^* contrast is increased after compartmentalization of iron oxides. Bowen *et al.* showed that the R_2^* sensitivity to iron oxide-labeled cells was 70 times better than for R_2 , and 3100 times better than R_1 (Bowen 2002). Phantom experiments performed at high field (9.4 T) showed that single HeLa ovarian tumor cells labeled with anionic maghemite nanoparticles (~ 6 pg Fe/cell) were visible on high-resolution gradient-echo (T_2^* -weighted) images but were barely detected on spin echo (T_2 -weighted) images (Smirnov 2006). Gradient-echo sequences are however sensitive to background signal voids due to blood vessels, imperfect shimming, air-tissue interfaces and endogenous iron. T_2 -weighted image are less affected to these confounding artifacts and provide more anatomical details than T_2^* -weighted images (Sykova 2011). A balanced steady-state free precession (b-SSFP) imaging sequence (e.g., FIESTA) has been used in several studies for monitoring the fate of isolated macrophages and cancer cells labeled with iron oxides. This sequence is characterized by a high signal to noise ratio and a high sensitivity to magnetic field inhomogeneities (Ribot 2011). Moreover, contrast is related to T_2/T_1 on these sequences, and is adapted for monitoring iron oxide-labeled cells (Scheffler 2003, Ribot 2011). Images with a high resolution are also needed to avoid partial volume effects. If the signal void induced by the SPIO-labeled cell is too small compared to the voxel size, it could not be detected on the MRI image (Magnitsky 2011). The magnetic field strength is also important. Increasing the external magnetic field generally shortens T_2^* relaxation times and facilitates the localization of iron oxide-labeled cells (Himmelreich 2009).

Iron oxides cell-based cell tracking studies were mainly performed to assess the delivery, the implantation and the migration of stem cells for cell-based therapies. As summarized in Table 4, the main imaging sites are the brain and the heart.

Table 4: Examples of MRI cell tracking studies for cell-based therapies using iron oxides

Labeling strategy	Cell type	Imaging site	Reference
Ferumoxides	Rat embryonic cardiomyoblasts	Rat heart	(Chen 2009)
Ferumoxides + Superfect [®]	Mesenchymal stem cells	Rat kidney	(Hauger 2006)
Ferumoxides + Protamine sulfate	Bone marrow stem cells	Porcine knee	(van Buul 2011)
Ferumoxides (SPIO) + PLL	Human cardiac-derived stem cells	Rat heart	(Terrovitis 2008)
	Neural stem cells	Mouse brain	(Ben-Hur 2007)
	Mesenchymal stem cells	Swine Liver	(Shi 2010)
	Macrophages	Mouse kidney	(Cai 2012)
Ferucarbotran (SPIO)	Neural stem cells	Mouse brain	(Politi 2007)
	Mesenchymal stem cells	Rat kidney	(Ittrich 2007)
Ferumoxtran-10 (USPIO) + FuGENE [®]	Embryonic stem cells	Rat brain	(Hoehn 2002)
1.63 μ m-MPIO	Hepatocytes	Mouse liver	(Shapiro 2006)
VSOP-C200	Embryonic stem cells	Rat brain	(Stroh 2005)
	Mesenchymal stem cells	Rat skeletal muscle	(Winkler 2008)
DMSA-coated anionic maghemite nanoparticles	Smooth muscle cells	Rat heart	(Riviere 2005)
Molday Ion Rhodamine B [®] (USPIO)	Neural stem cells	Mouse brain	(Berman 2011)
	Human glial precursor cells	Rat brain	(Gorelik 2012)

The monitorable time period depends of the proliferation rate, label retention and detection threshold. Finally, the stability of iron oxides is also important. In a rat model of thrombotic stroke, bone marrow stromal cells co-labeled with Ferumoxides and bromodeoxyuridine (BrdU) were implanted in the contralateral hemisphere. A hypointense area was first observed at the implantation site and dark spots were progressively observed in the lesion site at day 8 and persisted up to 47 days after injection. Immunohistochemistry using Prussian blue staining and anti-BrdU antibodies confirmed the presence of bone marrow stromal cells in the lesion 4 weeks after grafting (Jendelova 2003). Recent studies report shorter monitorable periods. Experiments on myocardial transplantation of SPIO-labeled stem cells showed that the persistence of MR signal voids was due to iron oxide

uptake by macrophages (Terrovitis 2008). A similar study compared the use of SPIO Feridex[®] (Ferumoxides) and the luciferase reporter gene for imaging transplanted cardiomyoblasts in the myocardium (Chen 2009). As shown in Figure 10, BLI signal decreased quickly to become absent on day 6 following injection, whereas MR signal void persisted up to 50 days. These examples illustrate that SPIO-based cell tracking is not adapted for long-term studies when the viability of injected cells might be affected after injection.

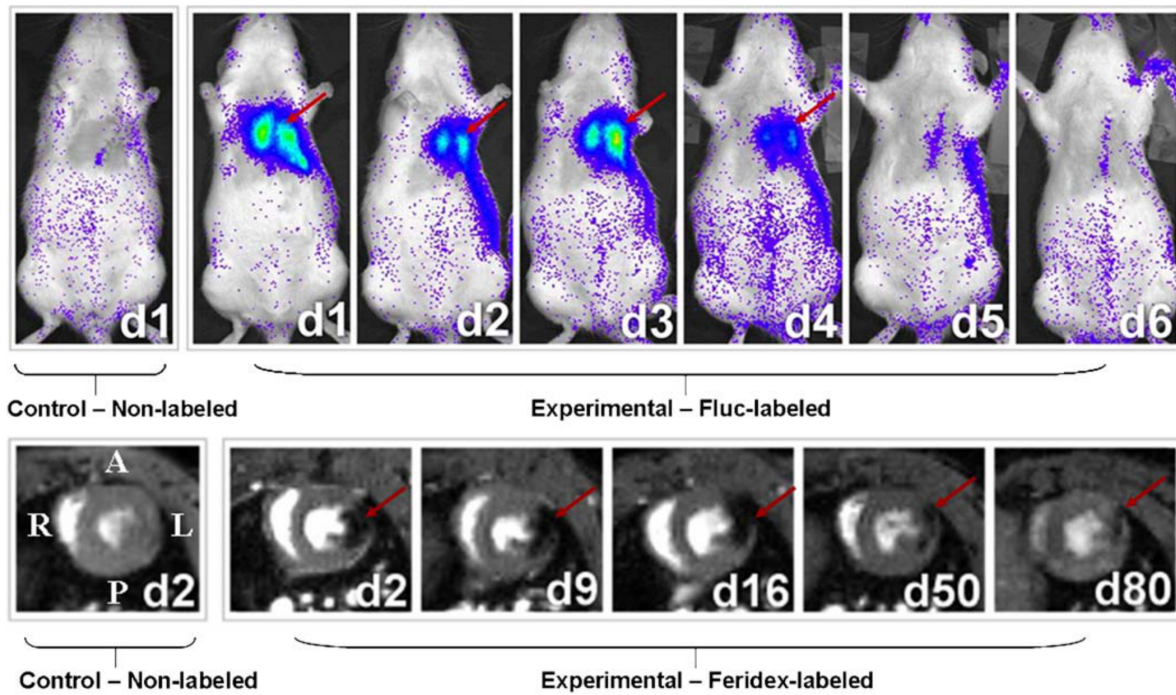


Figure 10: Comparison of SPIO labeling and the luciferase reporter gene for assessing the survival of transplanted cells. At day 50 post injection, hypointense spots were observed on MR scans due to the presence of Ferumoxides, whereas BLI showed that transplanted cardiomyoblasts did not survive (Chen 2009).

Assessing viability is important for noninvasive cell tracking applications. As shown with the previous example, the inability to differentiate live *versus* dead cells using direct SPIO cell tracking is an important drawback. However, several *in vitro* studies attempted to overcome this limitation by exploiting the differences in relaxivity between compartmentalized iron oxides (living cells) and uniformly dispersed iron oxides (dead cells) (Simon 2006, Henning 2009). Translating these results *in vivo* is challenging because the amount of iron oxides, the degree of interaction between protons and iron oxides (influenced also by the proton density), and the type of cell death (apoptosis *versus* necrosis) affect relaxation mechanisms (Henning 2009). Discrimination between live and lysed iron oxide-labeled cells was achieved *in vivo* using the b-SSFP sequence. It was shown that lysed iron oxide-labeled cells have a significantly higher signal compared to viable labeled cells. (Ribot 2012).

Although MRI is a rather insensitive method compared to other whole body imaging methods, the use of iron oxides dramatically increases the sensitivity of this imaging method (Serres 2012, Yang 2012). Sensitivity of cellular MRI is affected by the iron oxide content, field strength, signal to noise ratio, pulse sequence, and acquisition parameters (Liu 2009). Recent advances have led to the possibility to image single cells: single cell imaging was reported with the use of MPIO as well as with SPIO as labeling agents in the mouse brain (Shapiro 2006, Ribot 2011). Accordingly to Heyn *et al.*, no other whole body imaging method is capable of single cell tracking (Heyn 2005).

II.4.3. Contribution of iron oxide-based cell tracking studies in metastasis research

MRI cell tracking is used for studying interactions between immune cells such as dendritic cells, T cells and NK cells, and tumor cells in the context of cancer immunotherapy (Hong 2010, Ottobriini 2011). For instance, MRI was used to evidence the intratumoral homing of CD8⁺ cytotoxic T lymphocytes labeled with CLIO with a detection threshold of ~3 cells/voxel (Kircher 2003). In basic oncological research, MRI cell tracking was used for monitoring the fate of both cancer, and tumor-associated cells. For example, *in vivo* labeling with USPIO was used to verify the presence of TAMs, associated with a poor prognosis, in a transgenic mouse model of mammary adenocarcinoma (Daldrup-Link 2011). Alternatively, *in vitro* labeling approaches were used for monitoring the homing of macrophages to metastasized lymph nodes or the trafficking of endothelial progenitor cells to sites of tumor angiogenesis (Arbab 2006, Cho 2012).

With respect to metastasis, MRI cell tracking was used in several experimental models of brain metastases. The most important contribution of MRI cell tracking in characterizing the metastatic process was probably brought by the study of Heyn *et al.* In this work, GFP-expressing MDA-MB-231BR breast cancer cells labeled with MPIO were injected into the left ventricle of nude mice. The cell line, derived from the MDA-MB-231 breast cancer cell line, was established to generate brain macrometastases following intracardiac injection. Shortly after injection, signal voids were observed on FIESTA images. Assuming that one signal void was due to one labeled cell, authors estimated that 1-3 % of labeled cells were entrapped in the brain. The development of macrometastases from solitary cells was then followed with MRI up to one month post injection (Figure 11). When MPIO-labeled cells started to proliferate, the iron oxide content was diluted and signal void decreased with time. The authors showed that a vast majority of entrapped cells died (>90 %), whereas remaining cells were dormant (4.5 %) or proliferating (1.6 %) (Heyn 2006).

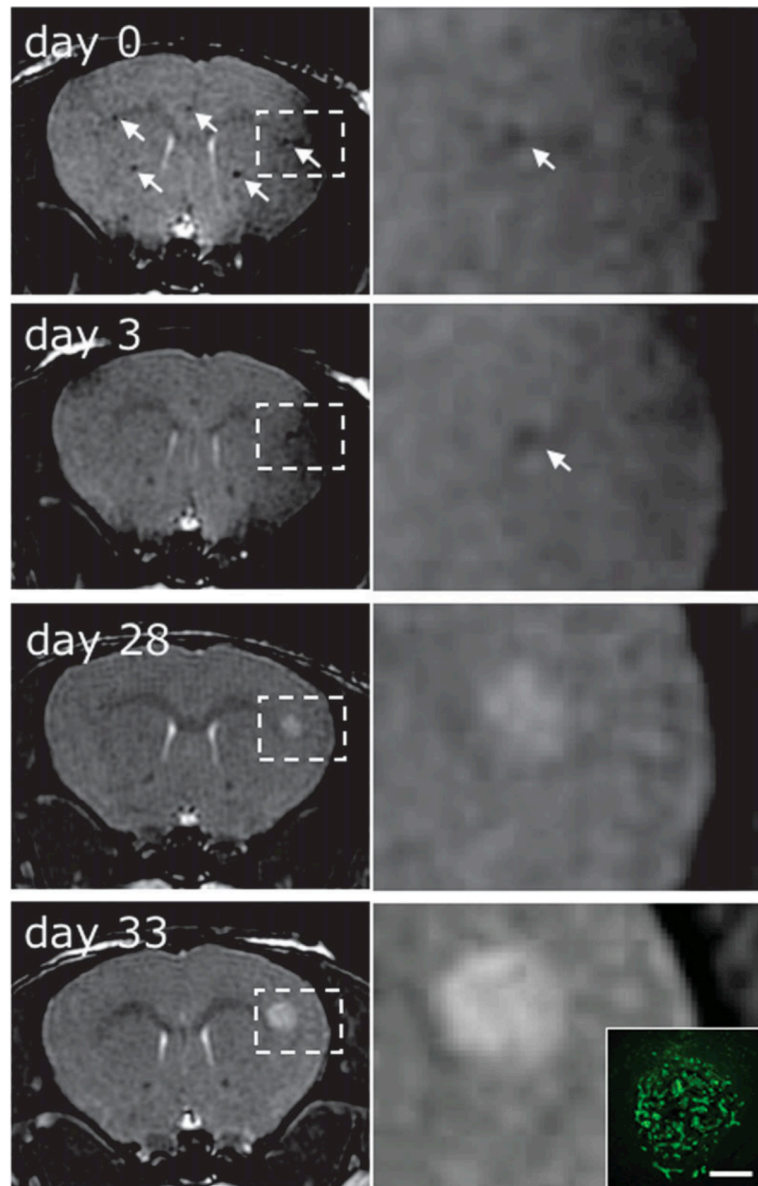


Figure 11: Longitudinal MR tracking of the growth of a single MPIO-labeled MDA-MB-231BR/EGFP cell into a tumor (hyperintense on the MR image). Of note, a majority of the signal voids that were initially observed disappeared with time. Persistent signal voids (not shown here) were shown to be due to non-proliferating MPIO-labeled cells. Confocal microscopy confirmed the presence of a GFP positive tumor (Heyn 2006).

In a related study, MDA-MB-231BR breast cancer cells labeled with Ferumoxides-protamine sulfate were imaged in the rat brain after intracardiac injection. One day following the injection of SPIO-labeled cancer cells, signal voids were observed in rat brains on T_2^* -weighted images at 3 T. Prussian blue iron staining and cytokeratin immunohistochemical staining confirmed the presence of SPIO-labeled cancer cells. Authors also observed a rapid loss of contrast with time, whereas macroscopic lesions appeared on T_2 -weighted images 2 weeks after injection (Song 2009). The lower resolution used in MR images compared to Heyn's study and the use of SPIO did not allow single cell tracking. Hence, quantitative T_2^*

imaging was proposed as an alternative to conventional anatomical images for assessing the distribution of SPIO-labeled cells. A significant shift to lower values was observed on T_2^* maps after injection of SPIO-labeled metastatic cancer cells compared to unlabeled cells (Song 2011). Concerning lymph node metastasis, cellular MRI was used for visualizing MPIO-labeled B16F10 melanoma cells in the mouse inguinal lymph node with a detection threshold of 100 cells (Foster 2008). Prior to imaging, repetitive *in vitro* iron quantification (using ICP-MS) showed that the iron oxide content was quickly diluted owing to intense proliferation. The iron levels dropped to 0 pg Fe/cell at day 3 after labeling, whereas cells were initially labeled with 35 pg Fe/cell (Foster 2008). These results are in agreement with a previous *in vitro* study that showed that SPIO-labeled Hela cancer cells lost their iron oxide content after 5 to 8 cell divisions (Arbab 2003b). FIESTA images showed however that signal voids induced by MPIO persisted with time during tumor growth. Control experiments excluded the possibility that hemorrhages and necrosis could be responsible of signal loss on MR images. Authors proposed that the contrast persistence reflected heterogeneity in cell proliferation, repartition of the magnetic particles between dividing cells, and/or cell death within the tumor (Foster 2008). The ability to distinguish proliferative *versus* quiescent cancer cells was further investigated in an experimental model of liver metastasis using MPIO-labeled B16F10 melanoma cells. Dormant cells appeared as persistent signal voids on MR images, whereas signal voids decreased with time when cells proliferated. In addition, macroscopic tumors were shown to appear hyperintense on T_2 -weighted and on b-SSFP images. Using MRI cell tracking of MPIO-labeled melanoma cells, it was shown that doxorubicin treatment inhibited tumor growth but did not affect the number of solitary dormant cells in the liver (Townson 2009). In another study, MRI cell tracking was used for imaging slowly proliferating melanoma cells labeled with iron oxides. Signal voids were attributed to iron-retaining melanoma cells but also to macrophages present in the tumor (Magnitsky 2011). To sum up, MRI cell tracking was shown to provide noninvasively some information on the number, the localization of metastases as well as on the proliferative status of metastatic cells (Perera 2012).

II.4.4. MR reporter genes

As a part of molecular imaging modalities, MR reporter genes have been developed to monitor noninvasively (trans)gene expression. In cell tracking studies, the use of MR reporter genes circumvents a major drawback of direct labeling strategies: the extrinsic nature of the signal. Based on their mechanism of contrast generation, MR reporter genes can be classified

in four main categories: iron-based, enzyme-based, plasma membrane-bound peptides and chemical-exchange saturation transfer (CEST)-based reporters (Lee 2012, Vandsburger 2012).

II.4.4.1. Iron-based strategies

Iron-based reporter genes constitute the most important category of MR reporter genes. These reporter genes code for proteins involved in iron homeostasis. Iron levels in cells are tightly controlled because free labile iron catalyzes the Fenton reaction that leads to the production of hydroxyl radicals (Vandsburger 2012). Iron uptake is mediated by the transferrin receptor (TfR), and iron storage depends on its intracellular binding to the ferritin protein. After intravenous administration of monocrystalline iron oxides covalently linked to human holo-transferrin, Weissleder *et al.* succeeded to visualize gliosarcoma cells stably transfected with the TfR on T_2^* -weighted images (Weissleder 2000). A similar strategy was recently used by Wang *et al.* to visualize the expression of therapeutic genes. In this study, they aimed to visualize breast cancer cells transfected with a retroviral plasmid encoding mouse endostatin and TfR. Mice bearing subcutaneous breast tumors presented a decrease in T_2 relaxation times in the tumor area after injection of USPIO conjugated to transferrin. Meanwhile, a decrease in tumor growth was observed owing to the expression of endostatin (Wang 2010).

Ferritin is the most studied MR reporter gene: it has been known for decades to decrease the T_2 of surrounding protons. This metalloprotein encompasses 24 light and heavy subunits and contains up to 4500 Fe^{3+} atoms (Figure 12) (Gossuin 2009, Carmona 2013). The amount of iron atoms is called the loading factor and influences the magnetic properties of the protein (Vymazal 1996, Vymazal 1998). The ferritin shell stores iron as ferrihydrite crystals ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$) conferring to the protein superparamagnetic properties. The transverse relaxivity of ferritin is nevertheless 2 orders of magnitude lower than that of iron oxide nanoparticles (Gossuin 2009).

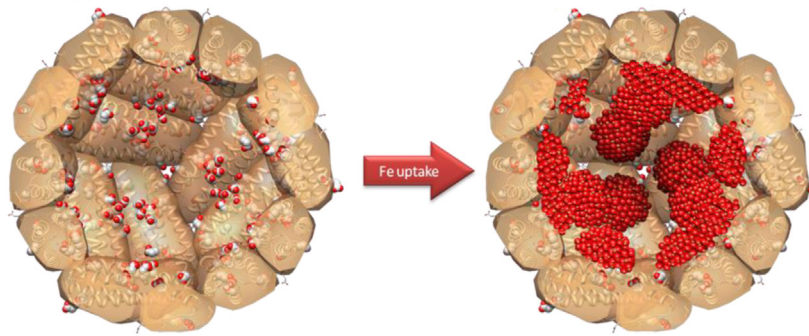


Figure 12: Cross-sectional view of the iron uptake by the ferritin protein (Carmona 2013)

Cohen *et al.* showed that ferritin heavy chain-overexpressing glioma cells implanted in the hind limb of mice induced MR contrast via a T_2 shortening effect (Cohen 2005). Genove *et al.* further evidenced the presence of hypointense regions on T_2 -, T_2^* -weighted images in the mouse brain striatum after stereotactic injection of adenoviral vectors containing transgenes for the light and heavy ferritin subunits (Genove 2005). In both studies, the overexpression of ferritin did not affect cell viability but enhanced cellular iron loading (Cohen 2005, Genove 2005). In fact, it was reported that TfR expression increases to compensate a decrease in the iron pool after ferritin overexpression (Cozzi 2000). Such mechanism opposes a possible “dilution effect”: ferritin content and its iron load are shared between daughter cells after division (Vandsburger 2012). Hence, ferritin was proposed for imaging metastatic lymph nodes after inoculation of highly proliferative melanoma B16F10 cells expressing the heavy human ferritin chain. As shown in Figure 13, median values calculated from cumulative T_2^* histograms were lower for ferritin-overexpressing tumors compared to control tumors (Choi 2012).

Ferritin is considered to be the most suitable MR reporter gene for the *in vivo* imaging of cell migration (Vandsburger 2012). For example, mouse subventricular zone progenitor cells were transduced *in situ* with adenoviral vectors encoding a chimeric ferritin molecule fusing both light and heavy subunits with a flexible polypeptide linker (Iordanova 2012). The migration of these cells to the olfactory bulb was imaged on T_2^* -weighted images. However, studies by Vande Velde *et al.* showed that ferritin-based approaches have low sensitivity (Vande Velde 2011, Vande Velde 2012).

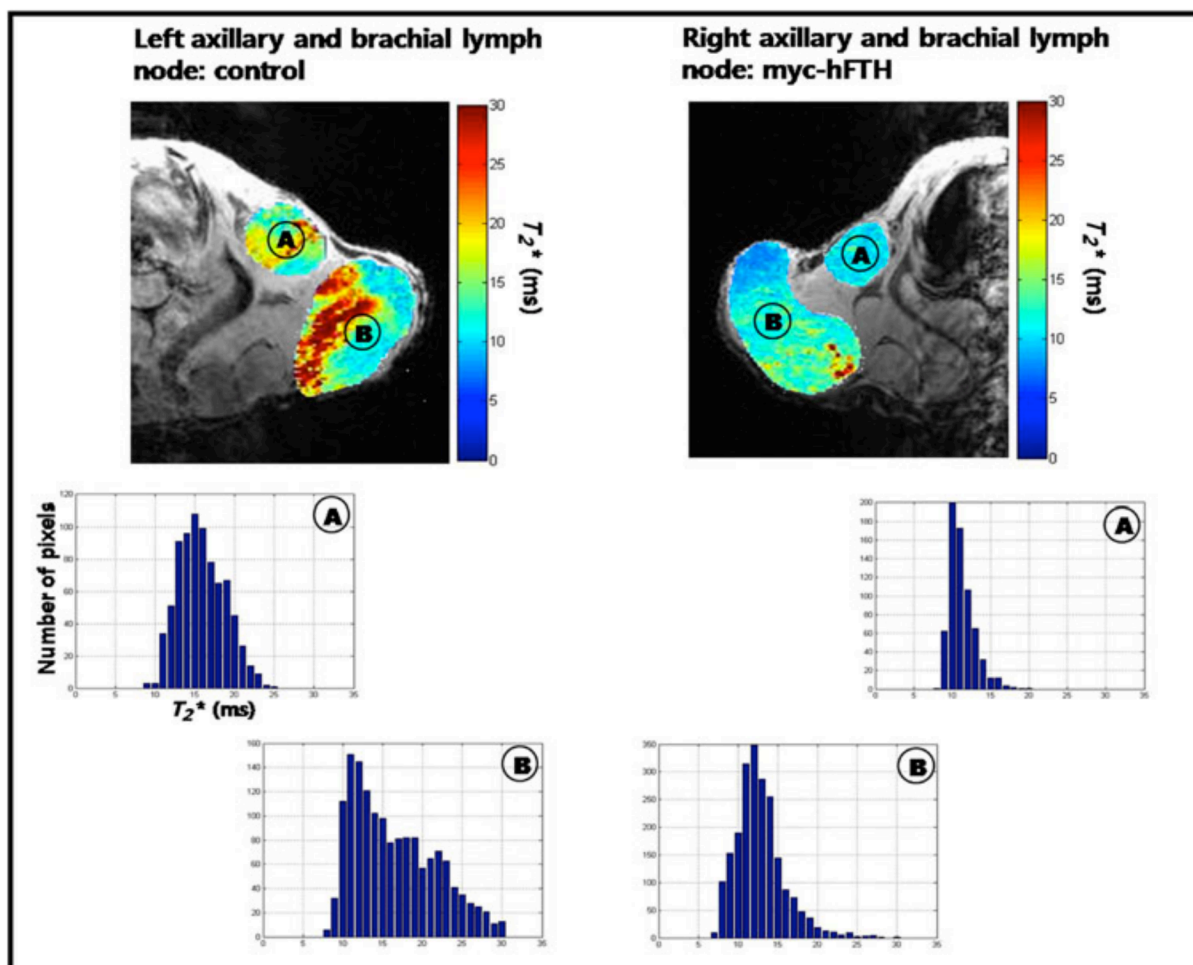


Figure 13: T_2^* maps and histograms of control and ferritin-expressing (myc-hFTH) B16F10 melanoma cells in mouse lymph nodes. The median T_2^* values in regions of interest A and B were lower in myc-hFTH B16F10 tumors compared to control tumors (Choi 2012).

Another protein, MagA, was proposed as a MR reporter gene (Zurkiya 2008, Goldhawk 2009). MagA is an iron transporter presumably involved in the magnetosome formation in magnetotactic bacteria. Although the role of MagA in iron oxide synthesis was recently debated (Uebe 2012), this protein was used by Zurkiya *et al.* to induce endogenous iron oxide synthesis in human embryonic kidney 293T cells. Five days after stereotactic injection in the mouse brain of MagA-expressing 293T cells, signal voids on T_2^* -weighted images were detected at the injection site, whereas GFP-expressing cells used as control did not produce any T_2^* contrast (Zurkiya 2008).

II.4.4.2. Enzyme-based strategies

The use of β -galactosidase for generating MRI contrast was initially proposed by Louie *et al.* β -galactosidase, encoded by the LacZ gene, is a common reporter protein responsible for the hydrolysis of lactose to galactose and glucose. In cells, the enzyme

catalyzes the transformation of analogous substrates such as X-gal into colored products (Ghim 2010). Louie *et al.* used a gadolinium-based substrate surrounded by a galactose group, EgadMe. The principle of the strategy was that in the presence of lacZ-expressing cells, EgadMe would be cleaved by β -galactosidase, and water molecules would then be allowed to interact with the gadolinium ions. Accordingly, it resulted in an increase in relaxivity through the inner sphere process as lacZ positive cells appeared hyperintense on T₁-weighted images (Louie 2000). Recently, gadolinium chelates conjugated to sugar moieties and tyrosine residues were used for the detection β -gal-expressing melanoma. The presence of tyrosinase, involved in the melanin synthesis of melanoma cells, allowed the polymerization and the accumulation of CAs, whereas β -gal released the sugar moiety thus producing contrast (Arena 2011).

Although T₁ reporter genes hold promises for cell tracking applications, protecting gadolinium ions with galactose turned out to be insufficient to suppress outer surface relaxation changes of such compounds (Gilad 2007b). In addition, knowing the concentration of CAs in transgenic cells *in vivo* may be problematic. Hence, data interpretation using enzyme-based MR reporter genes remains challenging (Gilad 2007b). Other approaches attempted to exploit other sources of contrast. In another study, the reaction of the histological staining compound 3,4-cyclohexenoescluletin β -D-galactopyranoside with ferric ammonium citrate in the presence of β -galactosidase was exploited to form paramagnetic iron chelates. After intratumoral injection of both reagents, signal voids were observed in lacZ-positive tumors on T₂*-weighted images (Cui 2010). A similar approach was recently applied using a fluorinated substrate. It allowed a dual ¹H/¹⁹F detection in xenografts of prostate tumors in immunodeficient mice (Yu 2012).

Tyrosinase is another example of enzyme-based reporter gene. As mentioned before, this enzyme is involved in melanin synthesis. Melanin is a paramagnetic radical with a high affinity for metallic ions. Although a recent study questioned the MRI contrast properties of melanin (Godechal 2013b), an *in vitro* study showed that breast cancer cells transfected with the tyrosinase gene displayed reduced T₁ values (Paproski 2011). Similar observations were also found in mouse fibroblasts and in human embryonic kidney cells (Weissleder 1997).

II.4.4.3. Plasma membrane-bound peptides

The use of intracellular reporter proteins implies that the DNA construct encoding the imaging probe must cross the cell membrane to gain access to the intracellular compartment. Conversely, cell-surface proteins are more accessible to imaging agents. Accordingly,

Tannous *et al.* engineered a transmembrane protein subject to biotinylation by the endogenous biotin ligase of mammalian cells. MRI or fluorescence imaging using streptavidin conjugated to magnetic nanoparticles or fluorochromes was used to image glioma cells expressing this biotinylated protein injected in mice. This method was also used with streptavidin–horseradish peroxidase controlling the activation of a peroxidase-sensitive gadolinium agent (Tannous 2006). Using streptavidin-magnetic nanoparticles, a two-fold reduction in T_2 values was observed in transgenic *versus* control tumors. With fluorescence-mediated tomography, a 15-fold increase in fluorescence signal was observed in transgenic tumors *versus* control tumors. Thus, this imaging platform has for advantage to allow a bimodal detection of cells either via a T_1 , T_2 shortening effect using MRI, or using fluorescence imaging (Tannous 2006). Alternatively, epitope-antibody interactions allow the specific visualization of molecular events. Using retroviral vectors, Chuang *et al.* transduced tumor cells with anti-polyethylene glycol (PEG) reporter membrane proteins (Chuang 2010). The reporter protein itself was a Fab fragment of a mouse anti-PEG monoclonal antibody fused to a transmembrane peptide. Transgenic tumors were imaged after intravenous administration of PEGylated probes (PEG-SPIO for MRI, pegylated ^{124}I agents for PET imaging, and a PEGylated optical imaging agent).

Multimodal imaging is the main advantage of cell membrane bound peptides but the detection of transgenic cells often requires the use of large tumor models (Vandsburger 2012). Recently, the longitudinal follow-up of transgenic embryonic stem cells (2.5×10^5) implanted in the infarcted myocardium was performed using a plasma membrane-bound reporter protein, expressing two antigens: hemagglutinin A and myc. SPIO-conjugated monoclonal antibodies against these antigens were used as imaging probes (Chung 2011) (Figure 14).

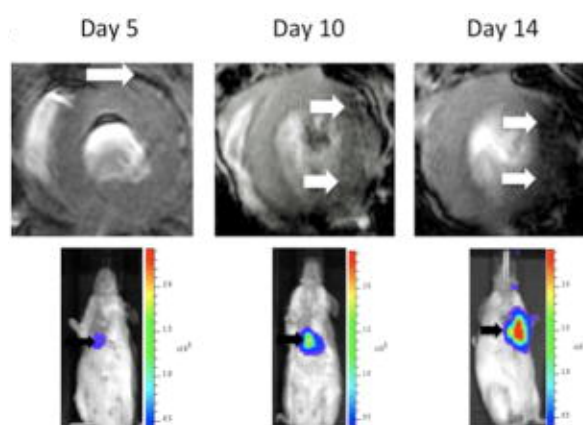


Figure 14: *In vivo* cardiac MRI (top) and BLI (bottom) of transplanted embryonic stem cells in the infarcted myocardium. This experiment shows that, after administration of SPIO targeting hemagglutinin A and myc, specific signal voids arising from transplanted cells expressing hemagglutinin A and myc antigens were produced (white arrows) (Chung 2011).

II.4.4.4. CEST agents

Protons in amide groups of polypeptides such as poly-*L*-lysine have a nuclear magnetic resonance frequency distinct from surrounding solvent water protons. This feature is exploited in chemical-exchange saturation transfer (CEST) MRI. It is based on the fact that the selective saturation of the amide proton signal of a reporter gene product interacting with surrounding water molecules attenuates the MRI signal from water protons. The “switchable” nature of CEST reporter agents compared to (super)paramagnetic CAs is one of the main advantage of this class of reporter genes (Gu 2012). Gilad *et al.* used a lysine rich-protein as a CEST reporter to visualize glioma cells in the mouse brain at 11.7 T (Figure 15) (Gilad 2007a). CEST MRI is however highly influenced by static magnetic field inhomogeneities and, its sensitivity is limited to the millimolar range (Lee 2012, Vandsburger 2012).

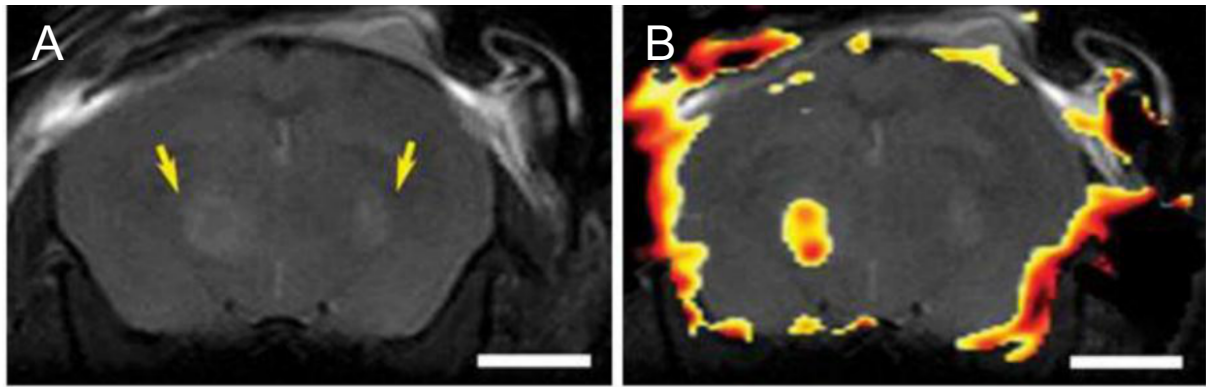


Figure 15: Chemical-exchange saturation transfer (CEST) MRI. (A) Anatomical image showing a lysine rich-protein-expressing tumor (left arrow) and a control tumor (right arrow) in the mouse brain. (B) Composite anatomical/CEST signal intensity-difference map showing the localization of the LRP-expressing tumor (Gilad 2007a, Lee 2012).

II.4.5. Summary

To summarize this section, numerous sources of contrasts are available for MR imaging. This diversity is well represented in MRI cell tracking studies. Table 5 summarizes the different MRI cell tracking approaches.

Table 5: Summary of MR labeling strategies for cell tracking applications

Labeling method	Advantages	Limitations
Paramagnetic CAs (e.g., Gd chelates)	• Straightforward interpretation of MR images (T_1 contrast)	• Poor internalization of Gd chelates • T_1 contrast is affected by the internalization process • Limited sensitivity • Potentially toxic (e.g. neurotoxicity for Mn^{++}, dechelation of Gd complexes) • Release of the label after cell death • Dilution of the label during cell division
Perfluorocarbons (^{19}F MRI)	• Absence of endogenous signal (“hot spot imaging”) • Quantification possible • Absence of toxicity	• Limited sensitivity • Release of the label after cell death • Dilution of the label during cell division
Superparamagnetic CAs (iron oxides)	• High sensitivity of detection under some specific circumstances (e.g., brain imaging)	• Presence of several hypointense confounding factors on T_2-, T_2^*-weighted images • Possible toxicity risks for labeled cells • Release of the label after cell death • Dilution of the label after cell division • Exocytosis of the label
MR reporter genes	• The MR reporter gene is passed to daughter cells during division	• Limited sensitivity

II.5. Optical imaging

II.5.1. Direct optical cell tracking methods

Optical labeling consists in the *ex vivo* delivery of nanoparticles carrying photoluminescent tags into cell of interest prior to their injection in small organisms. Imaging optically labeled cells is as sensitive as imaging radiolabeled cells, but it does not expose the host and the labeled cells to ionizing radiations (Bhirde 2011). However, one major limitation of the strategy is the poor penetration of light (at excitation and emission frequencies) in deep tissues. Therefore, the detection of optically labeled cells is limited to a few centimeters of tissue width (Bhirde 2011). Different optical imaging methods exist for direct cell tracking, each of these requiring a specific optical contrast agent. The main imaging methods are fluorescence, bioluminescence and photoacoustic imaging. Classical optical agents such as fluorescent proteins, organic dyes and fluorescent lanthanide chelates suffer from photo bleaching and metabolic degradation *in vivo*. In addition of being stable and nontoxic, optical CAs used for cell labeling must be photostable, highly photoluminescent (high quantum yield), and they must emit light at wavelengths comprised between 700 to 1300 nm in order to avoid light scattering by tissues. Below this range, light is absorbed by haemoglobin (Janowski 2012). Water molecules absorb light above 1300 nm (Janowski 2012). Finally, a large spectral shift between the emission and the excitation wavelengths (i.e., a large Stoke shift) is needed to minimize excitation light-induced background (Chen 2012, Janowski 2012). Quantum dots, upconversion nanoparticles and gold nanoparticles are the most popular optical agents in cell tracking studies. Aside from these nanoparticles, numerous other optical agents have been developed including polymer dots, nanodiamonds and dye-doped nanoparticles (Janowski 2012, Gao 2013).

II.5.1.1. Quantum dots

Among optical agents, quantum dots (QDs) are widely used nanoparticles for cell tracking applications. They were initially developed as stable fluorescent markers for immunofluorescence assays, DNA array technology and cell and animal biology (Michalet 2005), and later used for cancer imaging (Fang 2012, Pericleous 2012, Alam 2013). QDs are fluorescent semiconductor nanocrystals with a diameter of 2-10 nm (Fang 2012). These nanoparticles are generally constituted by a core made of CdSe or CdTe surrounded by a ZnS

shell (Rak-Raszewska 2012). They have a high quantum yield, a high molar extinction coefficient and a large Stoke shift (Bhirde 2011). These nanoparticles are not affected by photobleaching, and are characterized by a wide absorbance and a narrow emission band. The wavelength of emitted light varies with the size of the particles. Large QDs emit low energy red light, whereas small QDs emit high energy blue light. As a consequence, multicolor imaging of different cell lines labeled with different QDs is feasible (Smith 2010, Alam 2013). The simultaneous tracking of different cell lines using *in vivo* multiplex imaging is one of the most attractive advantages of QDs. For example, Lin *et al.* labeled murine embryonic stem cells with different QDs (emitting at 525, 565, 605, 655, 705, and 800 nm). QD-labeled cells were then subcutaneously injected in nude mice. Although different cell populations were simultaneously imaged, fluorescence intensity was different for each QD (Figure 16). The maximal emission intensity was achieved with QDs emitting in the near-infrared (NIR) range (Lin 2007). At NIR wavelengths (~ 800 nm), light scattering by tissues and background fluorescence are reduced (Alam 2013). Modifications of the shell composition and of surface coating can increase the signal intensity but they do not affect the emission range (Pericleous 2012). QDs were successfully used for labeling and imaging several cell types including mesenchymal stem cells, fibroblasts and cancer cells (Voura 2004, Ranjbarvaziri 2011, Damalakiene 2013).

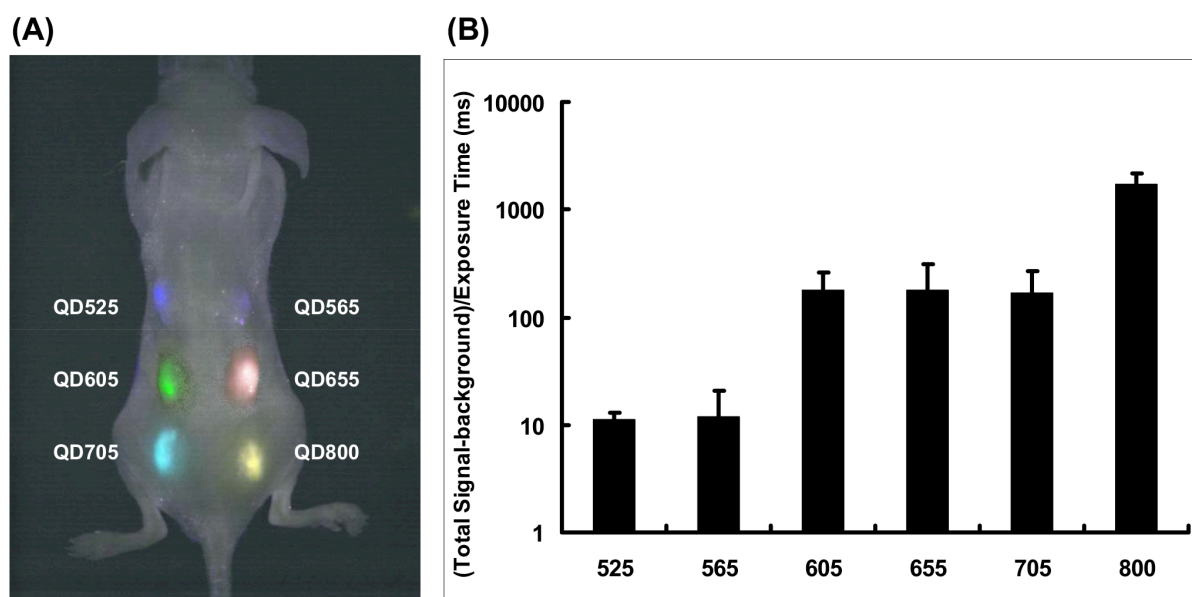


Figure 16: (A) Multiplex imaging of QD-labeled cells subcutaneously injected in nude mice. Numbers correspond to the emission wavelengths of QDs (nm) (B) Signal quantification shows that QDs emitting at 800 nm offer the highest fluorescence intensity (Lin 2007).

There are mainly three methods for labeling cells with QDs: passive labeling, facilitated delivery and active delivery. Passive labeling is nonspecific and involves electrostatic interactions between QDs and the plasma membrane (Liu 2010). For passive labeling, uptake mechanisms vary with the coating and incubation conditions. For instance, confocal microscopy studies showed that carboxyl-coated QDs can be internalized after nonspecific binding and the clustering of the nanoparticles near cationic sites on the plasma membrane, triggering their subsequent endocytosis. Proteins contained in the serum can also adsorb onto the surface of QDs and activate receptor-mediated endocytosis (Damalakiene 2013). Facilitated delivery refers to the use of molecules grafted to the QDs that allow the entry of the particles inside cells. These molecules can be cell-penetrating peptides (e.g., tat peptide), proteins (transferrin, antibodies, etc), all triggering endocytosis after ligation to specific receptors or smaller molecules (e.g., dopamine for labeling fibroblasts transfected with the dopamine receptor) (Delehanty 2009). Alternatively, lipid formulations, allowing the intracellular delivery of QDs after fusion with the cell membrane, were also used for labeling purposes (Al-Jamal 2008, Delehanty 2009). Active delivery refers to the use of electroporation or microinjection (Delehanty 2009).

Although QDs hold great promises for cell tracking applications, their potential cytotoxicity is a major concern. After internalization, QDs accumulate in lysosomes where they undergo acidic degradation (Karabanovas 2008, Damalakiene 2013). The cadmium core of the nanoparticles is obviously highly toxic for the labeled cells. However, coating acts as a physical barrier and cytotoxicity decreases with the number of surface layers (Pericleous 2012). Indeed, human mesenchymal stem cells incubated for 24 h with silica-coated QDs and cysteine as capping ligands had a cell viability of 90 %, whereas cells labeled with uncoated QDs showed significant cell death (70% cell viability) (Lai 2009). However, other studies reported that QD labeling was safe. Ranjbarvaziri *et al.* labeled human bone marrow mesenchymal stem cells with QDs using positively charged peptides (Ranjbarvaziri 2011). They did not observe adverse events on cell viability, cell differentiation and morphology after an incubation time of 96 h. Noh *et al.* labeled dendritic cells with QDs emitting in the NIR region (Noh 2008). It did not affect cytokine production or chemokine-dependent cell migration. Moreover, allogenic naive T cell activation and the uptake of exogenous antigens were not significantly different between QD-labeled and unlabeled dendritic cells. *In vivo* imaging was used to monitor the migration of QD-labeled dendritic cells from the footpad of mice towards the inguinal and popliteal lymph nodes. *In vivo* imaging results, confirmed by immunohistofluorescence, showed that the cells accumulated in lymph nodes 48 h post injection.

In addition of their potential toxicity, exocytosis of internalized QDs was reported. Furthermore, labeling cells with QDs suffers from the same major limitation than other direct cell tracking approaches, i.e., the dilution of the label with cell proliferation. Indeed, Rak-Raszewska *et al.* labeled embryonic stem cells (ESC) and kidney-derived stem cells (KSC) with QDs: two hours later, 85% of both cell lines were efficiently labeled and most QDs were found in late endosomes and lysosomes, but three days following labeling, the percentage of QD-labeled cells had fallen to 10% for ESCs and 40% for KSCs (Rak-Raszewska 2012). Conversely, both cell lines retained QDs after labeling for 3 days when treated with mitomycin C, a well-known inhibitor of cell proliferation. Conclusively, despite the potential toxicity risks and the loss of QDs with cell proliferation, this labeling strategy is suitable for short-term cell tracking studies in small animals.

II.5.1.2. Upconversion nanoparticles

Among optical agents, rare-earth-doped nanophosphor upconversion nanoparticles (UCNP) are worth mentioning. After the absorption of two or more photons, UCNPs emit light at shorter wavelengths than the excitation light. Hence, upconversion is an anti-Stokes process: the emitted light has higher energy than excitation light (Gao 2013). UCNPs are excited in the NIR region and emit light in the NIR or visible domain. The imaging depth is thus increased compared to classical fluorophores (Nagarajan 2011). The lack of autofluorescence background and the absence of photobleaching are other advantages of optical imaging with UCNPs. Wang *et al.* used UCNPs for labeling and tracking mesenchymal cells (Wang 2012a). After labeling, proliferation and differentiation were not affected by the nanoparticles, and no exocytosis of UCNPs was observed in labeled cells. After *in vitro* validation, nude mice subcutaneously injected with 10 to 10^4 UCNP-labeled cells were imaged using an *in vivo* upconversion luminescence imaging system. As few as ~ 10 labeled cells were detected.

II.5.1.3. Gold nanoparticles

Gold nanoparticles (Au NP) have versatile optical properties and were investigated for cellular imaging. To date, most imaging studies using Au NPs were performed *in vitro*. Before their use in optical imaging, gold nanoparticles have been used for a long time as CAs in electron microscopy. Interestingly, gold nanoparticles absorb X-ray very efficiently, making them interesting for cell tracking using micro-CT (Menk 2011). A cell labeling study

showed that Au NPs were not cytotoxic for labeled macrophages (Shukla 2005). Several subtypes of Au NPs exist based on their physicochemical properties. Although gold nanospheres were initially the only nanoparticles available, other types of Au NPs with different sizes and shapes, such as nanorods, nanoshells, and nanocages, have been developed to obtain optical properties suitable for biomedical applications (Cai 2008). For example, Au NPs have been used as intracellular labels for assessing *in vitro* the differentiation of embryonic stem cells using Raman spectroscopy (Sathuluri 2011). The quantum yield of Au NPs can be substantially increased by using a technique called Multiphoton-absorption-induced luminescence (MAIL), where Au NPs are simultaneously excited by two or more photons of red or NIR light (Gao 2013). Nagesha *et al.* successfully used MAIL for the *in vitro* imaging of embryonic stem cells labeled with gold nanoparticles (Nagesha 2007).

Fluorescence imaging lacks of spatial resolution in deep tissue imaging because of the scattering of excitation and emission lights. Photoacoustic imaging (PAI) was recently developed to overcome this limitation (Solomon 2011). This imaging modality is based on the conversion of light absorbed by endogenous chromophores in tissues (e.g., haemoglobin) into local heat produced by the nonradiative relaxation of the excited molecules (vibrations). Heat in turn causes a thermal expansion of the tissue, which produces acoustic waves that are collected using an ultrasound transducer (Solomon 2011). This hybrid imaging modality thus combines the high contrast and the spectroscopic-based specificity of optical imaging, and the high spatial resolution (for deep tissues) of ultrasound imaging (Gao 2013). The spatial resolution and the depth of imaging depend on the intensity of the ultrasound wave generated by photons (Solomon 2011). Modern instrumentation can achieve up to 30 mm depth with a 150 μm resolution (Kim 2010a). Cell tracking using PAI relies on the plasmonic properties of gold nanoparticles because cells do not have optical properties. Using PAI, Jokerst *et al.* visualized mesenchymal stem cells labeled with silica-coated gold nanorods with a resolution of 340 μm and a temporal resolution of 0.2 s. The *in vivo* limit of detection of Au-labeled mesenchymal stem cells in mouse hind limb muscle was 90 000 cells (Jokerst 2012).

II.5.2. Optical reporter genes

II.5.2.1. Fluorescence imaging

Fluorescence imaging played a pivotal role in molecular cancer imaging for assessing tumor response to treatment, tumor growth and/or metastatic dissemination. Green fluorescent protein (GFP) is one of the most commonly used cell tag. It was extensively used in cell

biology for studying cellular dynamics or for tracking proteins (using fusion proteins) (Bremer 2001). With regard to noninvasive cell tracking, GFP was used for monitoring *in vivo* the growth and the metastatic dissemination of murine melanoma B16F10-GFP cells. Using fluorescence imaging, a 60 μm diameter tumor was visualized at a depth of 0.5 mm, whereas a 1.8 mm tumor was detected at 2.2 mm depth (Yang 2000). However, *in vivo* fluorescence imaging is limited by autofluorescence and absorption of light signal produced by fluorescent molecules (fluorophores or fluorescent proteins) (Ray 2011). To overcome light scattering by tissues and to improve imaging depths, the development of fluorescent proteins emitting in the NIR domain is warranted but remains challenging (Chudakov 2010). Indeed, most of far-red fluorescent proteins have a lower brightness compared to GFP. A fluorescent protein called Katushka was proposed for whole body imaging owing to its high emitting intensity in far-red fluorescence. However, the excitation peaks of fluorescent proteins in general ($<590\text{ nm}$) diverge from the optimal “optical window” (700-1300 nm), which constitutes a main limitation of their use for cell tracking (Chudakov 2010). Tomographic reconstruction techniques have been used in fluorescence imaging to reconstruct 3D images and to improve the imaging depth (2-3 cm) (Condeelis 2010).

II.5.2.2. Bioluminescence imaging

Bioluminescence is a natural phenomenon observed in deep-sea organisms and in terrestrial animals, such as fireflies. It occurs via luciferases and photoproteins. Luciferase enzymes have been isolated from both marine (e.g., *renilla* luciferase) and terrestrial (e.g., firefly luciferase) organisms. In the presence of ATP and Mg^{++} , firefly luciferase (fluc) catalyzes the oxidation of *D*-luciferin in oxyluciferin. The reaction also generates CO_2 , inorganic pyrophosphate (PPi), and light (560 nm). Other luciferases isolated from *renilla* or *gaussia* are ATP-independent and smaller compared with fluc. Instead they require the use of coelenterazine, a substrate that is quickly degraded *in vivo* and has a limited biodistribution after injection. These luciferases emit a blue light ($\sim 480\text{ nm}$) that is strongly scattered by tissues. New red-shiftants of *renilla* luciferases are currently developed to overcome this limitation (Loening 2007). Currently, fluc is the most widely used luciferase in biological applications. Imaging of small animals containing fluc-expressing cells can start 10 to 15 minutes after the intraperitoneal injection of *D*-luciferin. The signal remains generally stable during 30 to 60 minutes (de Almeida 2011). Luciferase may be used as a constitutive reporter gene, allowing a semi-quantitative monitoring of the survival and growth of different cell lines including stem cells or cancer cells (Figure 17). Luciferase genes may also be driven by

specific promoter sequences and for determining the activity of transcription factors *in vivo* (Keyaerts 2012). Last, “split” luciferases allow the study of physical interactions between two proteins. As explained by Brogan *et al.*: “two likely interacting protein partners are each fused to N- or C-terminal domains of the firefly luciferase gene, which does not have catalytic abilities of its own, and co-transduces into a cell of interest. When the two interacting proteins are brought together through any kind of signal activation, they also bring together the N- and C-terminal halves, which reconstitutes the enzymatic abilities of luciferase” (Brogan 2012).

The main advantages of BLI are its high sensitivity and the absence of background signal. Unlike fluorescence imaging, external illumination is not necessary. As a consequence, there is no scattering by tissues of any excitation light and consequently no photon emission from tissues (autofluorescence). As aforementioned, light scattering by tissues is minimal at wavelengths between 700 to 1300 nm. Unfortunately however, the wavelength of photons emitted by fluc is out of this range (de Almeida 2011). Therefore, attenuation and scattering of the emitted light are the main drawbacks of BLI: signal intensity may be reduced by a factor 10 for each centimeter of tissue between the source and the skin surface (Huang 2012). Because planar imaging (i.e., 2D imaging) is the most common signal acquisition method, images generally lack of depth information, spatial resolution and quantitative accuracy (Solomon 2011). The development of the reporter gene luc2, with improved signal intensity compared to fluc, as well as new imaging and reconstruction methods aim to improve the resolution and the sensitivity of *in vivo* BLI (Kim 2010b, Solomon 2011).

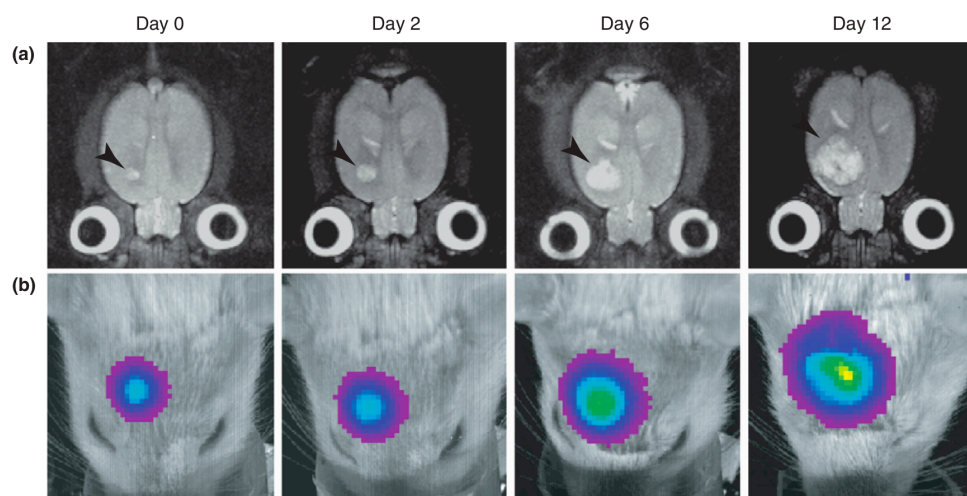


Figure 17: Use of BLI for monitoring tumor growth of luciferase-expressing 9L-luc gliosarcoma cells implanted in the rat brain (10^5 cells). Longitudinal monitoring of tumor progression using MRI (A) and BLI (B) (Rehemtulla 2000, Keyaerts 2012).

II.5.3. Summary

Optical imaging is an emerging and fast-evolving field for cellular and molecular imaging applications. Table 6 summarizes the main advantages and limitations of the optical labeling strategies discussed above.

Table 6: Summary of optical labeling strategies for cell tracking applications

Labeling method	Advantages	Limitations
Quantum dot	• High level of fluorescence • Low photobleaching • Tunable emission wavelength allowing multiplex imaging	• Potential toxicity • Exocytosis of the label • Release of the label after cell death • Dilution of the label during cell division
Upconversion nanoparticles	• Excitation by NIR light • No autofluorescence nor photobleaching	• Release of the label after cell death • Dilution of the label during cell division
Gold nanoparticles	• Versatile imaging platform (optical imaging, X-ray imaging, PAI)	• Release of the label after cell death • Dilution of the label after cell division
Fluorescent proteins (e.g., GFP)	• Numerous biological applications • Signal is preserved during cell division if the transgene is stably expressed • Signal is only produced by living cells	• Optical properties out of “the optical window” (700-1300 nm) • Autofluorescence
Luminescent proteins (e.g., luciferase)	• Numerous biological applications • Signal is preserved after cell division • Signal is produced only by living cells if stably expressed • High sensitivity • Absence of background signal	• Light scattering by tissues • Lack of spatial resolution

II.6. Radionuclide imaging

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) are main radionuclide techniques used for *in vivo* imaging. SPECT uses low energy γ -emitting isotopes (e.g., ^{123}I , ^{111}In and $^{99\text{m}}\text{Tc}$), whereas PET is based on the

annihilation coincidence detection of probes carrying positron-emitting radioisotopes (e.g., ^{18}F). SPECT allows the simultaneous detection of multiple radionuclides emitting γ -rays with different energies (Kircher 2011). Multiple biological events can thus be recorded simultaneously using different radiotracers. PET does not allow this but has however numerous advantages compared to SPECT. First, it is more sensitive than SPECT because of the geometry of the scanners. In PET imaging systems, the object is surrounded by a ring of detectors, whereas SPECT data acquisition is limited by one single physical collimator that rotates around the object to acquire a tomographic image. Quantification is a main advantage of SPECT/PET systems. With regard to signal quantification, PET is more robust than SPECT (Hicks 2012).

II.6.1. Direct cell radiolabeling

SPECT/PET radiolabels with a long half-life are preferred for direct cell tracking applications because such tracers extend the monitorable period of time. Several SPECT radiotracers, including ^{111}In -oxyquinoline (^{111}In -oxine) ($t_{1/2}=2.8$ days) and $^{99\text{m}}\text{Tc}$ -exametazime (also known as $^{99\text{m}}\text{Tc}$ -HMPAO), have initially been used for the *ex vivo* labeling of leukocytes. ^{111}In -oxine is a FDA-approved drug used for white blood cell scintigraphy (Kircher 2011). This drug enters into cells via a lipophilic chelator. Once inside, ^{111}In -oxine binds to intracellular proteins (Kircher 2011). $^{99\text{m}}\text{Tc}$ -HMPAO was used for imaging inflammation processes in patients with suspected infections (Love 2004). Although Technetium 99m has a shorter radioactive half-life than Indium 111 ($t_{1/2}=6$ h), it can be used at higher doses to improve the visualization of small objects. $^{99\text{m}}\text{Tc}$ -HMPAO was used to assess stem cell trafficking in acute and chronic myocardial infarction (Gu 2012). This drug is mainly eliminated via biliary excretion, and shows bowel activity. Therefore, cell tracking using this agent in abdominal areas might be problematic (Kircher 2011). Direct strategies were also used for monitoring cellular immunotherapy. For example, scintigraphy served to monitor the migration of ^{111}In -oxine-labeled tumor infiltrating lymphocytes in melanoma patients (Fisher 1989). More recently, tumor-specific CD8 cytolytic T lymphocytes (CTL) targeting hemagglutinin (HA) were labeled with ^{111}In -oxine. After a lymphodepletion (mouse irradiation) known to improve the anti-tumor T cell activity, ^{111}In -oxine-labeled CTLs were shown to accumulate in the tumors of mice bearing HA-expressing tumors (Figure 18) (Pittet 2007). ^{111}In -oxine was shown to be a biocompatible labeling agent with no adverse effect on cell viability or functions (Kircher 2008). However, Brenner *et al.* observed a significant reduction in cell viability (30 % of cell death compared to unlabeled cells) and in their

migratory potential after radiolabeling hematopoietic progenitor cells with 30 MBq ^{111}In -oxine for 60 minutes in serum-free medium (Brenner 2004).

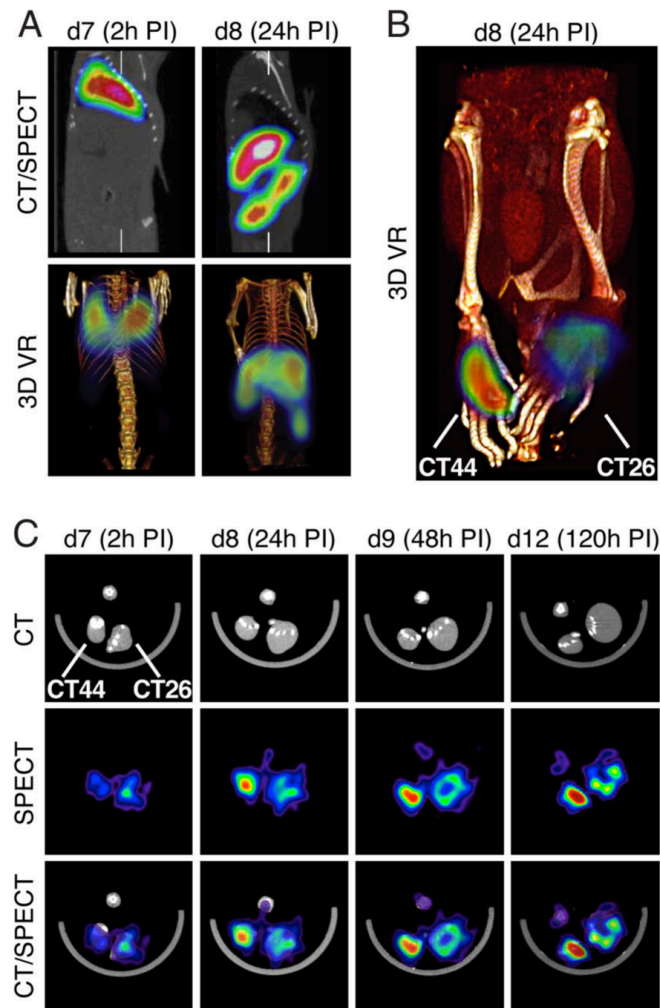


Figure 18: In vivo SPECT/CT monitoring of the distribution of CTL targeting HA. (A) SPECT/CT and 3D virtual rendering (3D VR) images of mice after injection of ^{111}In -labeled CTLs. (B) 3D VR images of HA-specific CTL accumulation in HA+ (CT44) and HA- (CT26) tumors. (C) Specific accumulation of ^{111}In -labeled CTLs in HA+ tumors (CT44). PI, postinjection (Pittet 2007).

2-Deoxy-2- ^{18}F fluoroglucose (^{18}F -FDG) and ^{64}Cu -pyruvaldehyde-bis-(N^4 -methylthiosemicarbazone) (^{64}Cu -PTSM) are the main PET radiotracers used in cell tracking studies (Kircher 2011). The short radioactive half-life of ^{18}F ($t_{1/2}=110$ min) does not allow the long-term monitoring of cells, whereas ^{64}Cu -PTSM ($t_{1/2}=12.7$ h) might be useful for relatively long-term and repeated observations (Wu 2013). As a glucose analog, ^{18}F -FDG enters into cell via glucose transporter (GLUT)-facilitated transport, after which it is phosphorylated by a hexokinase into ^{18}F -deoxyglucose-6-phosphate. The latter cannot be catabolized by glycolytic enzymes and will accumulate within the cell. ^{18}F -FDG is used in the clinic for the detection of glycolytic tumors (Gatenby 2004). ^{64}Cu -PTSM is a lipophilic complex that

passively diffuses across the cell membrane. Once inside the cell, the stable complex $^{64}\text{Cu}(\text{II})$ -PTSM is reduced to a labile $^{64}\text{Cu}(\text{I})$ -PTSM complex. The released copper ions are retained within the cells because of charges, whereas neutral PTSM leaves the cell (Adonai 2002). Adonai *et al.* labeled C6 rat glioma cells with ^{64}Cu -PTSM or ^{18}F -FDG, and measured their biodistribution after intravenous injection. Radiolabeling uptake was better with ^{64}Cu -PTSM than with ^{18}F -FDG. This study concluded that ^{64}Cu -PTSM was superior to ^{18}F -FDG for cell tracking studies, even if an active efflux of both agents was observed (Adonai 2002). As alternatives, other PET tracers have been developed for cell labeling applications. For example, *N*-succinimidyl 4- ^{18}F -fluorobenzoate (SFB) was used for labeling and tracking bone marrow-derived dendritic cells (Olasz 2002). Labeled cells were injected into the footpad of mice and their migration to the draining lymph node was monitored 4 hours after implantation using PET imaging. In a recent study, the engraftment of human circulating progenitor cells into the rat infarcted myocardium was assessed using PET imaging after *ex vivo* labeling with either ^{18}F -FDG or hexadecyl-4- ^{18}F -fluorobenzoate (^{18}F -HFB) (Zhang 2012). The ^{18}F -HFB-CPC signal in the infarcted area at 2 h postinjection was stronger than that of ^{18}F -FDG-labeled cells, whereas ^{18}F -FDG activity was found in non target areas. Efflux of ^{18}F -FDG from labeled cells was set forward to explain this observation. ^{18}F -HFB is a lipophilic long-chain ester and was used to label stem cells by absorption into the cellular membranes (Ma 2005).

In addition to the inherent radioactive decay of radiolabels that limits the monitorable time window, direct radiolabeling approaches share the same limitations with other direct cell tracking approaches, i.e., the dilution of the label with cell division and the uptake of released radiolabels by macrophages. Efflux of radiotracers from labeled cells was observed for several agents. Such effect can lead to an underestimation of the number of labeled cells in a given tissue area (Kircher 2011). It is of further note that the exposure of labeled cells to ionizing radiations may be harmful. It was however shown that mesenchymal stem cells, often used in cell tracking studies, possess robust antioxidant and DNA repair mechanisms (Chen 2006).

II.6.2. PET/SPECT Reporter genes

Owing to the intrinsically high sensitivity of PET/SPECT imaging, the use of PET/SPECT reporter genes constitute an important class of molecular and cellular imaging modalities. Herpes Simplex virus thymidine kinase 1 (HSV1-tk) and other variants are the most frequently used PET/SPECT reporter genes (Kircher 2011, Brader 2013). Once

expressed by cells of interest, the corresponding proteins phosphorylate radiolabeled nucleoside analog substrates. Among them, ^{18}F -FEAU, ^{18}F -FHBC and ^{124}I -FIAU are widely used. Phosphorylated radiolabels carry a negative charge and cannot passively leave intact cells (Kircher 2011). The HSV1-tk reporter gene was used by Koehne *et al.* for tracking the accumulation of Epstein-barr virus (EBV)-specific T cells in EBV-positive tumors (Koehne 2003). Repeated intravenous injections of ^{124}I -FIAU or ^{131}I -FIAU in tumor-bearing mice allowed the repeated imaging (PET and scintigraphy) of the accumulation of HSV1-tk-transduced T cells. The use of HSV1-tk reporter gene provides information on cell function, for example in the context of TGF- β signaling pathway in the metastatic cascade of tumors. With regard to breast cancer bone metastases, TGF- β stimulates the osteolytic destruction of the bone matrix adjacent to tumor. Serganova *et al.* used TGF- β -sensitive reporters coding notably for HSV1-tk for assessing *in vivo* the location and progression of murine breast cancer bone metastases and TGF- β signaling using microPET (Serganova 2009). HSV1-tk also phosphorylates drugs such as ganciclovir (causing their intracellular accumulation) and may therefore be used as a suicide therapeutic gene (Brader 2013). The dual role of HSV1-tk as a reporter and a therapeutic gene was exploited in a clinical study involving glioblastoma patients (Jacobs 2001). Four days after the intratumoral injection of liposome-HSV1-tk complexes, a ganciclovir treatment was initiated. HSV-1-tk gene expression was monitored with ^{124}I -FIAU PET imaging, and the treatment response was repeated MRI, ^{18}F -FDG and carbon-11-labeled methionine scans. In one patient, ^{124}I -FIAU was found to accumulate at the site of HSV1-tk vector injection. In this patient, ^{18}F -FDG PET evidenced a response to ganciclovir in a portion of the tumor (Jacobs 2001). Despite the important sensitivity of HSV1-tk reporter genes, the development of HSV1-tk-based imaging methods is limited by the potential immunogenicity of the gene and by mutagenicity risks linked to the nucleoside substrates (Bonini 1997, Johnson 2001). Alternatives have been developed. They include the use of vectors encoding the sodium-iodine symporter, otherwise naturally expressed in the thyroid, stomach and salivary gland. The expression of the symporter in cells to be tracked can be imaged using ^{123}I , $^{99\text{m}}\text{Tc}$ for SPECT and ^{124}I for PET. Alternatively, engineered cells overexpressing the D2R dopamine receptor or the somatostatin receptor can be imaged with PET/SPECT using their respective radioligands (Kircher 2011).

II.6.3. Summary

Conclusively, the use of PET/SPECT reporter genes offers the advantages of a high sensitivity of detection. On another hand, direct SPECT/PET radiolabeling strategies suffer

from the same limitations than other direct labeling methods but in addition expose radiolabeled cells to ionizing radiations. Moreover, the monitorable time period is limited by the radioactive decays of the radiolabels.

III. Contribution of electron paramagnetic resonance for quantifying iron oxides in MRI studies^{*}

III.1. NMR versus EPR

Historically, electron paramagnetic resonance (EPR) [or equivalently electron spin resonance (ESR)] and nuclear magnetic resonance (NMR) were discovered at the same period of time. In 1944, Zavoisky first described the EPR phenomenon (Zavoisky 1945, Salikhov 1998). A little bit later, Bloch and Purcell discovered the NMR phenomenon (Bloch 1946, Purcell 1946). In 1973, Lauterbur published the original idea of applying magnetic field gradients in three dimensions to create NMR images (Lauterbur 1973). Since that time, MRI has been extensively developed and has revolutionized medical imaging. In other respects, the first *in vivo* EPR experiments were carried out in 1975 (Feldman 1975, Swartz 2003) and the first EPR imaging (EPRI) experiment was reported in 1979 (Hoch 1979, Eaton 1998). In comparison with the flourishing applications of MRI for the diagnosis and treatment of human diseases, the development of *in vivo* EPR and EPR imaging has been rather slow. There are several reasons for that, especially linked to the considerable technical challenges that make *in vivo* EPR difficult (Swartz 2003, Gallez 2004b).

There are few fundamental differences between the principles of electron and nuclear magnetic resonance as NMR- and EPR-related techniques share similar basic principles. However, the differences in physical and chemical properties of the resonant species (unpaired electrons *versus* nuclei with net spin) lead to profound differences in the techniques that are used to record the spectra or to reconstruct an image (Swartz 2003, Gallez 2004b). There are three major differences between EPR and NMR: i) the size of the magnet moments, which fixes the ratio between the frequency and the magnetic field; ii) the very short relaxations times of the electron spins compared to the nuclear spins; iii) the limited amounts of paramagnetic materials that are present in most biological systems.

The gyromagnetic ratio of an unpaired electron is much larger than that of a proton, so the resonance frequency/magnetic field ratio for the electron is 28 GHz/T *versus* 42.5 MHz/T for the proton. As a consequence, standard EPR spectrometers operate at much higher frequencies (microwaves) and lower fields than NMR spectrometers: the most usual commercial EPR spectrometers operate at 9 GHz (X-Band) for a magnetic field around 0.34

^{*} This section is adapted from the review: Danhier P, Gallez B. Electron Paramagnetic Resonance (EPR): a powerful tool to support Magnetic Resonance Imaging (MRI) research. *In Preparation*

T. At this high frequency, the non-resonant absorption of the electromagnetic radiation by the liquid water presents serious problems, and limits the sample size to 1 mm. Classical *in vitro* EPR experiments on cells are carried out in thin capillary tubes, and *in vivo* experiments at 9 GHz are limited to pharmacokinetic studies on the tail vein of mice (Mader 1993, Gallez 1996a). Larger aqueous samples or animals can be studied only by reducing the operating frequency, for example by using 1 GHz spectrometers (L-Band) (Berliner 1985, Subczynski 1986, Hutchinson 2003). At 1 GHz, the depth of penetration is of about 1 cm, but with a reduced sensitivity.

A second major difference between NMR and EPR is related to the relaxation times. While longitudinal T_1 and transversal T_2 relaxation times of protons are in the range of 0.1 to 1 second (Krishna 2001), electronic spins return to the equilibrium after excitation much more rapidly (ns- μ s). This feature has two consequences. First, EPR spectra in biological systems are mostly obtained through continuous wave (CW) experiments. Instead of applying an energy pulse and recording the transient response as done in NMR and MRI, the sample is continuously irradiated with a low-power microwave radiation while the magnetic field is gradually increased so that electron spins come into resonance. So far, only few groups succeeded in addressing the challenges of time-domain EPR *in vivo* (Subramanian 2003, Subramanian 2004, Subramanian 2008). The second consequence of short EPR relaxation times is that EPR imaging requires the application of magnetic gradients that are much larger than those used in MRI (Kuppusamy 2003).

The third but not the last difference between both MR techniques relies on the nature of the material that is studied. Compared to the 100 molar concentration of endogenous protons available for NMR, there is a lack of sufficient amounts of naturally occurring paramagnetic materials for EPR. Except for melanin that can be easily detected in biological samples (Vanea 2008, Godechal 2011, Cesareo 2012, Godechal 2012, Godechal 2013a), endogenous paramagnetic species are present in insufficient amounts to be detected by *in vivo* EPR. The short half-life of most free radicals involved in physiology (such as nitric oxide) or in toxicology (such as superoxide or hydroxyl radicals) is another source of difficulty. These reactive species are usually detected using a spin-trapping strategy by the formation of a stable spin adduct after reaction with a nitron (Timmins 2003). Generally, for *in vivo* and *in vitro* EPR biological studies, a stable paramagnetic material has to be introduced in the biological system to be detected. Very interestingly, the paramagnetic reporter may provide unique information on its environment such as *in vitro* and *in vivo* oxygenation (Liu 1993, Gallez 2004a, Khan 2007, Vikram 2007, Krishna 2012), redox status (Swartz 2007, Matsumoto 2011), pH (Gallez 1996b, Khramtsov 2004, Driesschaert 2012, Dhimitruka 2013)

or microviscosity (Halpern 1999, Kempe 2010). For instance, the interaction of biradical oxygen molecules with paramagnetic probes (e.g., nitroxyl radicals or nitroxides) results in modification of the linewidth of the EPR probe. This principle was used in several studies for assessing the oxygen consumption of cells *in vitro* and *ex vivo* (Figure 19) (Jordan 2002, Sonveaux 2008).

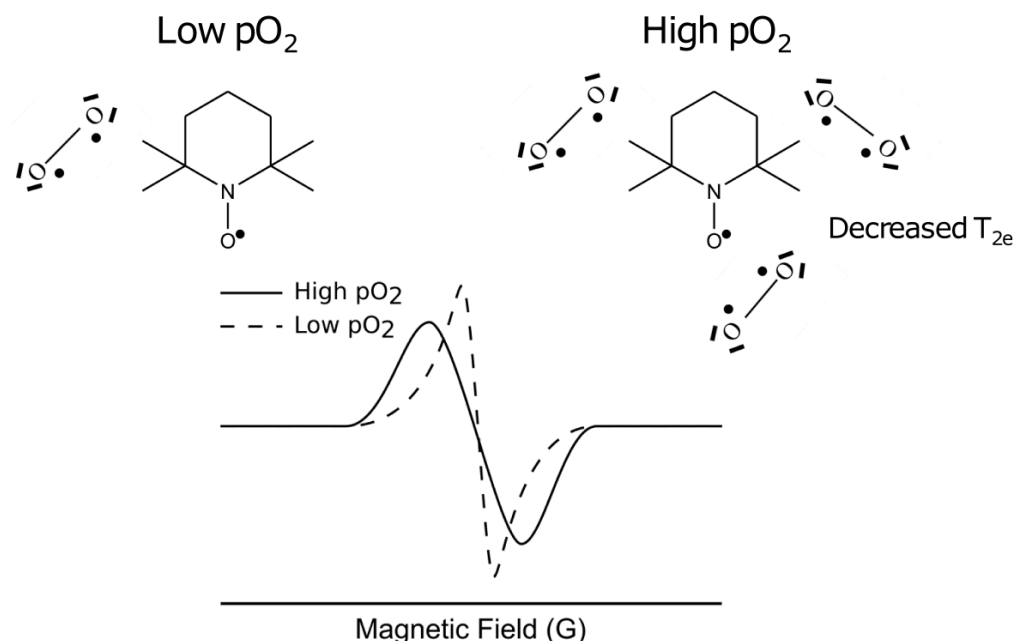


Figure 19: For oximetry studies, electronic transversal relaxation times (T_{2e}) of nitroxides are affected by the presence of surrounding oxygen molecules. Therefore, the linewidth of the EPR spectrum of a nitroxide is proportional to the $[O_2]$ of the sample.

For those not familiar with EPR spectroscopy, it should be acknowledged that the main parameters derived from EPR spectra are the g -values (analogous to the chemical shift in NMR), the electron-nuclear hyperfine coupling constants (analogous to nuclear spin-spin coupling constants in NMR), linewidths and intensities (Symons 1991). Because EPR spectra are usually displayed as first derivatives, linewidths are conveniently measured as the distance between points of maximum and minimum slope. Intensities are often measured as the amplitude of each line, but it is preferable to use the double integration of the signal to determine the concentration in a paramagnetic species.

III.2. Iron oxide quantification using EPR

Signal voids induced by iron oxides in T_2 -, T_2^* -weighted images may be confounded by several physiological events or artifacts linked to the presence of air cavities, blood

vessels, bone-tissue interfaces and hemorrhages (van den Bos 2006, Himmelreich 2009). Generally, the accumulation of iron oxides in the tissue of interest is confirmed by histochemistry using Prussian Blue staining (Schroeter 2004), but this technique is qualitative and not quantitative. The measurement of the concentration of iron oxide particles in tissues represents an important step for the qualification of new CAs (Levy 2011). To achieve that goal, radioactive ^{59}Fe -SPIO can be used, which requires the cumbersome chemical synthesis of radiolabeled nanoparticles (Weissleder 1989). Inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES) are alternatives that provide a sensitive quantification of elemental iron in tissues (Yancy 2005, Huang 2011, Dadashzadeh 2013). However, these methods are unable to discriminate endogenous iron from exogenous iron oxide nanoparticles. In this respect, EPR spectroscopy is an invaluable tool because the EPR spectrum exhibits different lines for the different chemical forms of iron. At room temperature, iron oxides contained in SPIO exhibit a broad and intense EPR spectrum that can easily be differentiated from free Fe^{3+} (Figure 20). Of note, the contribution of ferritin to the EPR spectrum of iron oxides was described to be negligible at room temperature (Levy 2011). A comparison study demonstrated that *ex vivo* EPR spectroscopy was superior to ICP-OES for profiling iron oxide biodistribution across tissues (Chertok 2010). Historically, one of the first applications of EPR for such application was performed on the liver of septic mice that had received SPIO administration (Fujii 2002). While new (U)SPIO particles are currently extensively designed for molecular imaging and theranostics (combining diagnosis and treatment using the same platform), *ex vivo* EPR spectroscopy is meant to be a reliable tool to monitor iron oxide nanoparticles after injection. Recent applications in this area demonstrated the usefulness of EPR to determine the concentration of USPIO targeted at E-selectin in the context of the MRI detection of inflammation (Radermacher 2009). By combining EPR and MRI, it was demonstrated that PEGylated USPIO functionalized by a mimetic molecule of the natural ligand of E-selectin (tetrasaccharide, sialyl Lewis X) could be a useful molecular MRI marker for the non-invasive diagnosis and monitoring of inflammation (Radermacher 2009). Interestingly, it was even possible to use low-frequency EPR to monitor directly *in vivo* the accumulation of these USPIO in inflamed tissues. Such EPR and MRI data further support the interest for active targeting through grafting particles with targeting agents compared to ungrafted ones. More recent studies used a similar approach to qualify USPIO targeting dying cells (Radermacher 2010, Radermacher 2012). On T_2 -weighted images, USPIO covered with hexapeptides targeting phosphatidylserine were shown to induce a signal loss in apoptotic areas within tumors. The same type of particles were shown to constitute an earlier MR biomarker of

tumor cell death (after cytotoxic treatments) compared to diffusion-weighted (DW)-MRI and choline measurement by ^1H MRS. EPR spectroscopy was used in these studies to validate MR data (Radermacher 2010, Radermacher 2012). It is likely that this particular application of EPR may be extended to many iron oxide particle platforms for the validation of new CAs. Concerning cell tracking applications, EPR was used in several studies for assessing the iron oxide content in cells (Wilhelm 2002, Billotey 2003, Smirnov 2004). Although it has mostly been used for iron oxide quantification, EPR was also used in one study in combination with magnetometry and relaxometry to elucidate the mechanism of T_2 -shortening induced by monocrystalline iron oxide particles (Bulte 1999a).

SPIO biodistribution

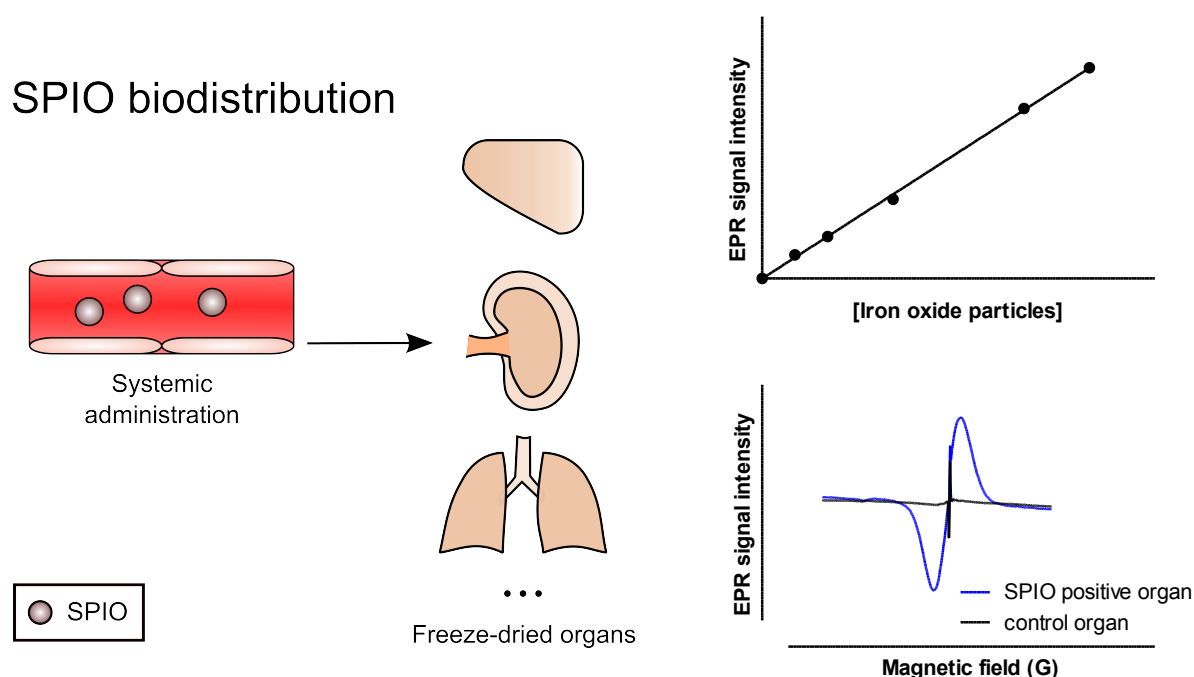


Figure 20: EPR as a quantification method of the iron oxide content in tissues. At room temperature, iron oxides (e.g., SPIO) exhibit a typical EPR spectrum, whereas endogenous iron in biological samples does not. There is a linear relationship between iron oxide concentration and EPR signal intensity.

Chapter II: Aims of the thesis

The first aim of this work was to implement MRI cell tracking using iron oxides for monitoring the fate of metastatic cancer cells. To address this question, it was first mandatory to optimize the labeling conditions. A first part of the study describes the use of EPR for quantifying the intracellular iron oxide content after magnetic labeling (Figure 21) (Danhier 2012). EPR was validated in comparison with routinely used techniques, namely ICP-MS and Perls' Prussian blue-based colorimetry.

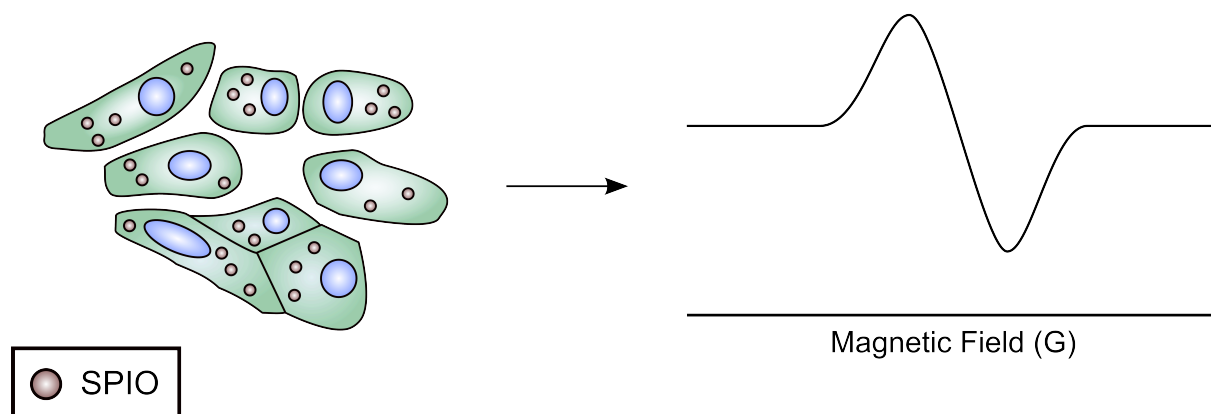


Figure 21: Use of EPR for the quantification of intracellular iron oxides (Danhier 2012).

Based on the validation of the method, we next questioned the ability of MRI to track iron oxide-labeled cancer cells using experimental and spontaneous models of metastasis (Danhier 2013b). This direct strategy was compared with the widely used indirect cell tracking strategy of bioluminescence imaging of luciferase-expressing cancer cells. EPR was used for measuring *ex vivo* the iron oxide content in tissues (Figure 22). Advantages and limitations of both approaches were also discussed.

In a third part of the study, MRI cell tracking was used for monitoring the entrapment of magnetically labeled metastatic cancer cells in the mouse brain shortly after intracardiac injection (experimental metastasis) (Danhier 2013c). In addition, the usefulness of EPR for accurately quantifying the number of cells in a defined tissue was tested.

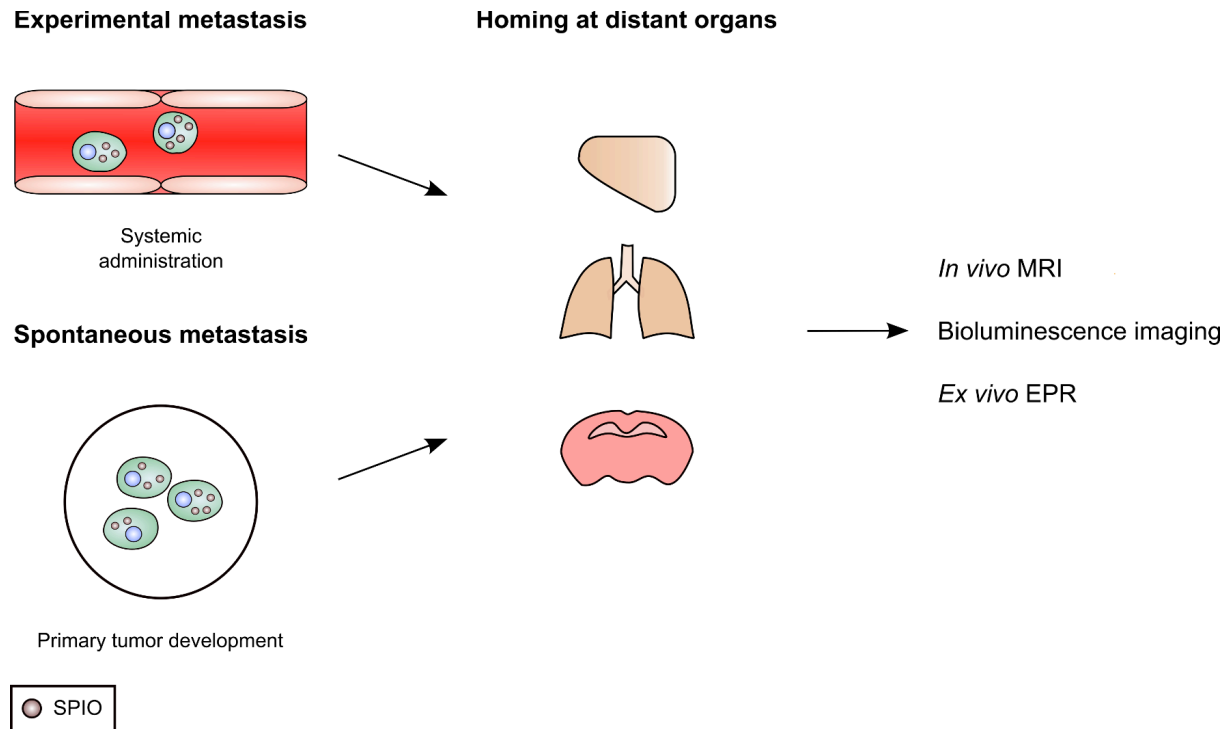


Figure 22: Use of magnetic cell tracking approaches (MRI and EPR) and BLI for assessing the fate of metastatic cells at distant organs.

Despite it provides information on the localization of cells of interest, MRI cell tracking does not give information on cell function and survival. Metastasis however is naturally an inefficient process with the vast majority of cells dying when released in the blood stream after detachment from the primary tumor. In the context of future imaging applications, we finally addressed the metabolic consequences of cell detachment (Figure 23) (Danhier 2013a).

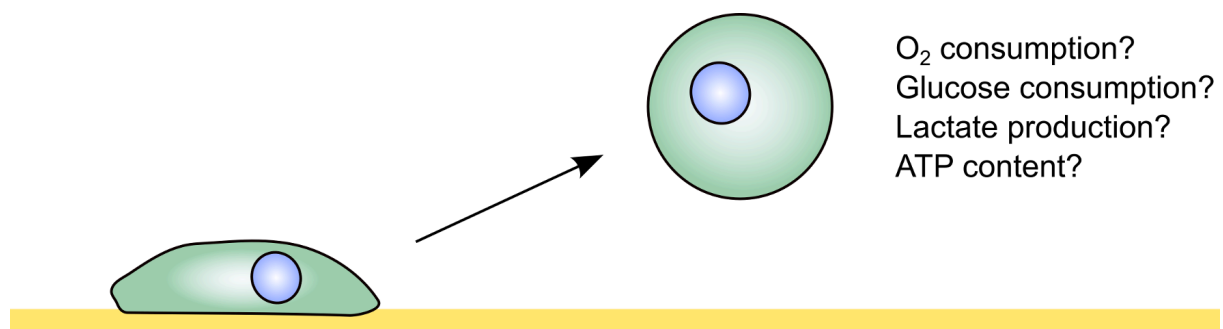


Figure 23: Influence of detachment on cell metabolism (Danhier 2013a).

Chapter III: Results

I. Electron paramagnetic resonance as a sensitive tool to assess the iron oxide content for MRI cell labeling studies

Pierre Danhier, Géraldine De Preter, Sébastien Boutry, Isabelle Mahieu, Philippe Leveque, Julie Magat, Vincent Hautfroid, Pierre Sonveaux, Caroline Bouzin, Olivier Feron, Robert N. Muller and Bernard Gallez

Optimizing labeling procedures is a first prerequisite step for iron oxide-based cell tracking studies. In this regard, quantification of intracellular iron oxide nanoparticles after passive labeling is mandatory. The aim of this study was to validate electron paramagnetic resonance for the quantification of iron oxides after cellular internalization. This study also allowed the determination of optimal labeling conditions for the subsequent studies.

Electron paramagnetic resonance as a sensitive tool to assess the iron oxide content in cells for MRI cell labeling studies

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ABSTRACT: MRI cell tracking is a promising technique to track various cell types (stem cells, tumor cells, etc.) in living animals. Usually, cells are incubated with iron oxides (T_2 contrast agent) in order to take up the particles before being injected *in vivo*. Iron oxide quantification is important in such studies for validating the labeling protocols and assessing the dilution of the particles with cell proliferation. We here propose to implement electron paramagnetic resonance (EPR) as a very sensitive method to quantify iron oxide concentration in cells. Iron oxide particles exhibit a unique EPR spectrum, which directly reflects the number of particles in a sample. In order to compare EPR with existing methods (Perls's Prussian blue reaction, ICP-MS and fluorimetry), we labeled tumor cells (melanoma and renal adenocarcinoma cell lines) and fibroblasts with fluorescent iron oxide particles, and determined the limits of detection of the different techniques. We show that EPR is a very sensitive technique and is specific for iron oxide quantification as measurements are not affected by endogenous iron. As a consequence, EPR is well adapted to perform *ex vivo* analysis of tissues after cell tracking experiments in order to confirm MRI results. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: EPR; cell labeling; iron oxide particles

1. INTRODUCTION

MRI cell tracking using iron oxide particles is a promising technique for assessing the fate of various cell lines after injection in a living organism. Numerous applications, such as stem cell follow-up in cell-based therapy (1–4) or cancer cells (5–7) in fundamental research, have been described. Intracellular iron quantification assays are essential for evaluating the initial iron oxide loading, optimizing the yield of iron uptake (8–10), and measuring the dilution of the iron oxide particles with cell proliferation (11). All these steps are crucial and can seriously affect cellular MRI studies. For example, iron quantification studies have shown that B16F10 melanoma labeled with MPIO lose all their iron content after three days (12). This experiment allowed prediction of the time window for tracking cells of interest by MRI. Eventually, a quantification method able to discriminate exogenous iron oxide particles from endogenous iron should be particularly interesting for *ex vivo* analysis of organs after cellular MRI studies in order to confirm previous MRI results.

Until now, the two main techniques used for assessing intracellular iron oxide content are spectrophotometric techniques (especially based on Perls' Prussian blue reaction) and inductively coupled plasma mass spectroscopy (ICP-MS) (13). The first one has the advantage of being implementable at low cost but may suffer from a lack of sensitivity whereas ICP-MS is expensive but highly sensitive. Perls' reaction-based colorimetry and ICP-MS measure both endogenous and exogenous iron, meaning that these two methods are not suitable for *ex vivo* analysis as described above. Many other iron oxide

particles are also grafted with fluorophores (for example MPIO commercialized by Bangs or VSOP from Ferropharm). Fluorescence of such particles is mainly used in microscopy to address the localization of the particles in the cells or tissues (6,14), but can also be used for quantifying indirectly the iron content (using fluorimetry). All these techniques are summarized in Fig. 1.

Electron paramagnetic resonance (EPR) is a spectrometric technique which is used to study free radicals and (super) paramagnetic molecules. A very important characteristic of EPR is its high sensitivity (owing to the gyromagnetic ratio

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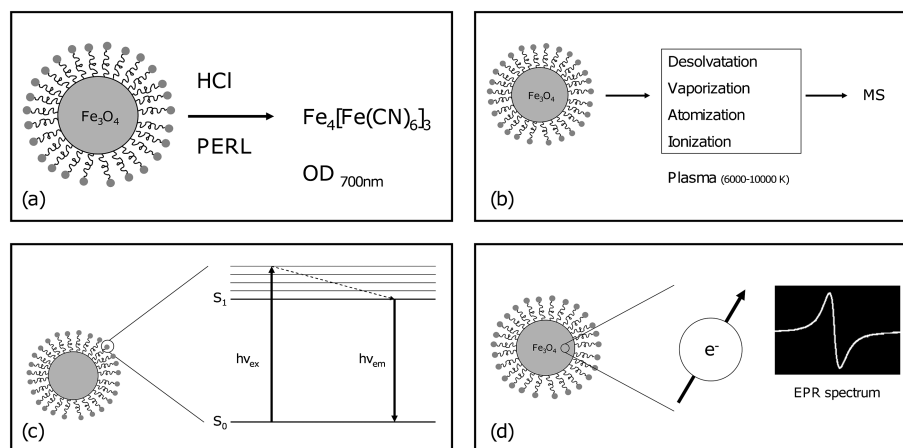


Figure 1. Methods used to quantify iron oxides in cell labeling. Perls' reaction-based colorimetry (a) and ICP-MS (b) measure the total iron content in the sample, while fluorimetry (c) requires the presence of a grafted fluorophore on the coating of the particle. Electron paramagnetic resonance is specific for the iron oxide core of the particles (d).

of the electronic spin which is much higher than that of proton). Iron oxides are superparamagnetic and present a typical EPR spectrum as represented in Fig. 2. Previously, EPR has already been described for studying iron oxide particles, mainly to characterize their physicochemical properties (15,16) or for SPIO distribution studies in tissues after systemic injection (17–19). This technique was also proposed by Wilhelm *et al.* for iron oxide quantification in macrophages (20). The goals of the present study were (i) to develop EPR for cell labeling experiments, and (ii) to compare the performances of EPR with the three classical methods (Perls' reaction-based colorimetry, ICP-MS and fluorimetry).

2. RESULTS AND DISCUSSION

2.1. Labeling Localization

Magnetic labeling using Molday ion rhodamine B (MIRB) of three cell lines (B16F10-luc melanoma, RENCA-luc renal adenocarcinoma and 3T3 fibroblasts) was assessed using fluorescence microscopy in order to confirm that further iron quantification studies correctly reflect intracellular iron content. Microscopy is essential to avoid any iron oxide quantification bias (particles adhering to plasma membranes or plates). All

cell lines incorporated MIRB particles efficiently (Fig. 3), with iron oxide particles localized in endosomes in the cytosol of the cells. Using microscopy, the labeling yield appeared to be higher for 3T3 cells than for RENCA-luc and B16F10-luc cells. The influence of serum deprivation was also evaluated. No clear effect of serum on the uptake yield was evidenced on the basis of microscopy observations, except for B16F10-luc, which incorporated fewer particles when the cells were incubated with MIRB particles in the presence compared with in the absence of serum.

2.2. Limit of Detection and Limit of Quantification

Several calibration curves were performed to determine the sensitivity of the different techniques (EPR and the pre-existing methods). The sensitivity of ICP-MS was not assessed since it is already well known that this technique is highly sensitive (detection limit lower than 1 ppm) (13). Limits of detection (LOD) and quantification (LOQ) were calculated using the following formulas: $LOD = 3SD/a$ and $LOQ = 10SD/a$ (where SD is the standard deviation and a is the slope of the calibration curves). As shown in Fig. 4, a linear relationship between optical density and iron concentration was observed for Perls' reaction-based colorimetry technique. However, the Lambert–Beer relationship was not preserved at lower concentration. This phenomenon has already been observed in a previous study (13) and was attributed to solubilization of the Prussian blue colloid at low concentration. For LOD and LOQ determination, a threshold value of $0.5 \mu\text{g Fe ml}^{-1}$ (beginning of linearity) was added to the previous formula. Therefore the LOD and LOQ for the Perls' reaction-based colorimetric technique were respectively 0.676 and $1.088 \mu\text{g Fe ml}^{-1}$.

Concerning fluorimetry, the emission of fluorescence from rhodamine B grafted to the MIRB particles was measured. The LOD and LOQ for this technique were 0.407 and $1.357 \mu\text{g Fe ml}^{-1}$. Finally, the EPR LOD and LOQ were, respectively, 91 and $304 \text{ ng Fe ml}^{-1}$. It is important to note that the volumes used for EPR experiments were low ($75 \mu\text{l}$), meaning that we were able to detect quantities of iron oxide as low as 6.8 ng . All LOD and LOQ of all techniques are summarized in Table 1.

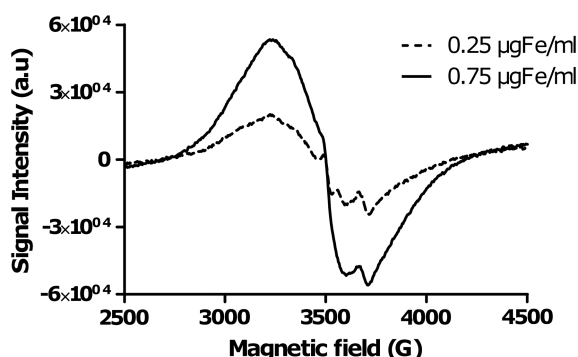


Figure 2. Typical electron paramagnetic resonance spectra of iron oxide (Molday ion rhodamine B particles) recorded at two different concentrations.

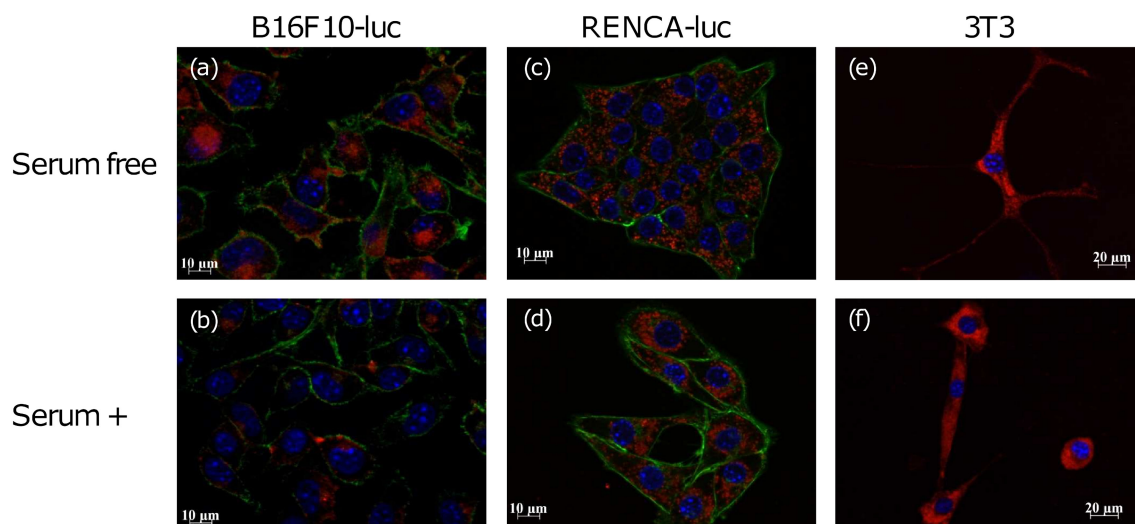


Figure 3. Microscopic images of cells labeled with Molday ion rhodamine B particles (MIRB; 100 $\mu\text{g Fe}/2\text{ ml}/9.6\text{ cm}^2$). The upper panels show the cells after overnight incubation in a serum-free medium and bottom panels after overnight incubation in the presence of serum. Three different cell lines were used: B16F10-luc melanoma cells (a, b), Renca-luc renal carcinoma cells (c, d), 3 T3 fibroblasts (e, f). Membranes are in green (phalloidin-FITC, not used for fibroblasts), nuclei in blue (Hoechst 33342), and MIRB particles (rhodamine B) in red.

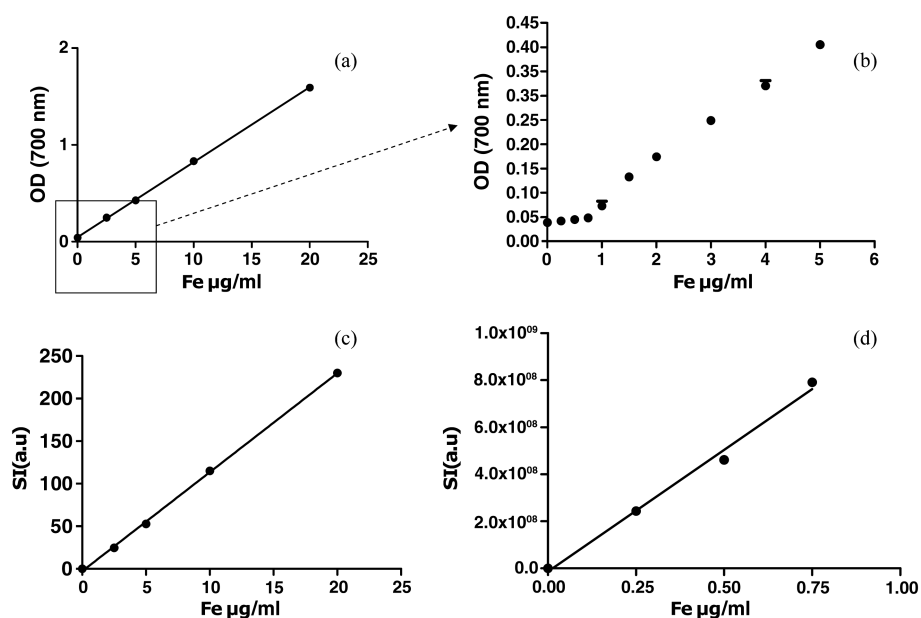


Figure 4. Calibration curves obtained for Perls' reaction-based colorimetry (a, b), fluorimetry (c) and electron paramagnetic resonance (EPR) (d). Note the differences of range in the x-axis (concentration), reflecting the difference in sensitivity of the methods. The highest sensitivity was found using EPR spectroscopy (c). Note also the absence of linearity at low concentrations using Perls' reaction-based colorimetry (b) (mean $\pm$ SEM, $n = 3$ for b).

Table 1. Limits of detection and quantification (LOD and LOQ) of the different techniques

Method	LOD	LOQ
Electron paramagnetic resonance	91 ng Fe ml^{-1}	304 ng Fe ml^{-1}
Perls' reaction-based colorimetry	0.676 $\mu\text{g Fe ml}^{-1}$	1.088 $\mu\text{g Fe ml}^{-1}$
Fluorimetry	0.407 $\mu\text{g Fe ml}^{-1}$	1.357 $\mu\text{g Fe ml}^{-1}$ (!Auto-fluorescence)
ICP-MS	<1 ppm (not assessed) (13)	Not assessed

2.3. Comparison of Iron Quantification Methods

To determine the reliability of EPR as a quantitative tool for measuring the content of iron oxide incorporated into cells, we compared the results obtained using EPR vs other methods above the LOQ of all methods where endogenous iron cannot interfere with the measurement. For this purpose, 3T3 cells were incubated overnight with full medium in the presence of MIRB particles ($28.5 \mu\text{g Fe}/2 \text{ ml}/9.6 \text{ cm}^2$). The next day, labeled cells were collected and intracellular iron content was measured with the four methods. Figure 5 shows that the average iron content per cell did not differ with the method used. Note that fluorimetry tended to slightly underestimate the amount of particles in cells. This was probably due to auto-fluorescence of cell preparations, as the values presented are corrected for the fluorescence measured in the absence of the particles.

2.4. Illustrative Application: Kinetics of Iron Oxide Uptake by Different Cell Lines

Kinetic experiments of MIRB uptake were performed in order to check whether EPR could be a useful method for optimizing labeling protocols. As shown in Fig. 6, B16F10-luc cells incorporated up to 7 pg Fe per cell while RENCA-luc cells incorporated 10–13 pg Fe per cell. Two interesting features are also displayed in this figure: the presence of serum dramatically decreased the yield of labeling and a plateau was observed after 24 h. The influence of serum was not so clear with fluorescence microscopy results, and this observation highlights the relevance of using quantitative and sensitive techniques as EPR for setting up the correct parameters for cell labeling procedures. In this case, the absence of serum is important to improve the yield of labeling and an overnight incubation in the presence of MIRB particles was found sufficient to obtain a maximal incorporation of iron oxide particles.

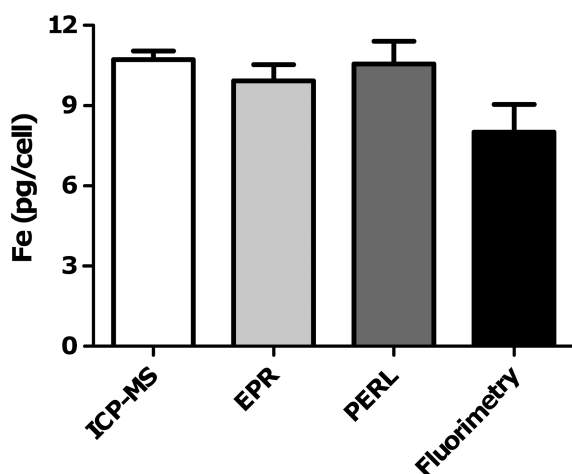


Figure 5. Comparison of techniques as tools for the quantification of iron oxide uptake in 3T3 cells. The techniques compared were ICP-MS, electron paramagnetic resonance, Perl's reaction-based colorimetry and fluorimetry. Bar graphs are expressed as picograms per cell (mean $\pm$ SEM, $n=3$ for a–c; $n=6$ for d). A Kruskal–Wallis test showed no statistical differences between the four methods ($p > 0.05$).

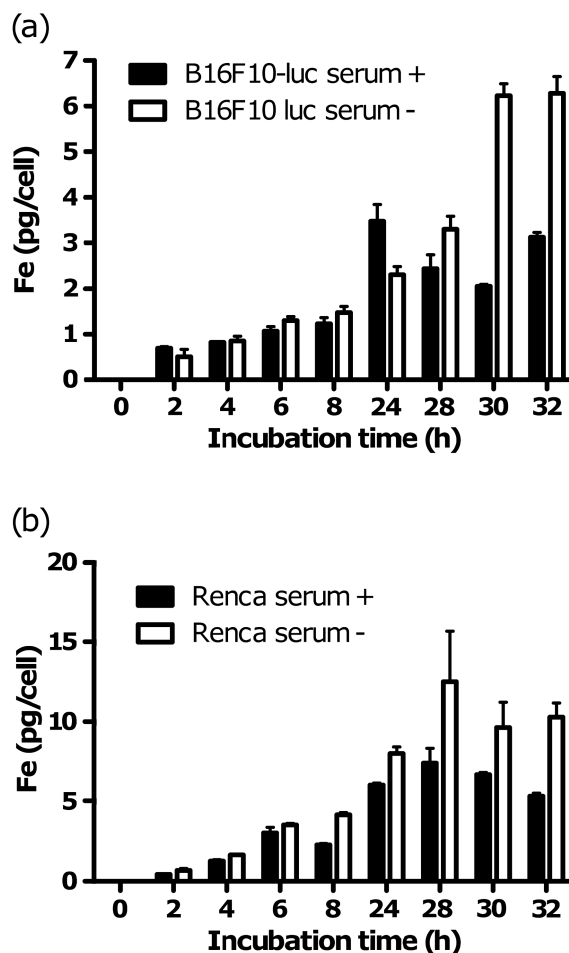


Figure 6. Kinetics of Molday ion rhodamine B uptake in B16F10-luc cells (a) and RENCA-luc cells (b). The experiments were carried out in serum or serum-free conditions. The iron oxide content of cells was estimated using electron paramagnetic resonance spectroscopy. Note the important influence of serum on particle uptake and on the plateau reached after 24 h of incubation (mean $\pm$ SEM, in triplicate).

3. CONCLUSIONS

We have evidenced in this present study that EPR is a highly sensitive technique for iron quantification for cell labeling studies when compared with all other available techniques: the limit of detection of EPR is about 7.5 times higher than that of the Perl's reaction-based colorimetry technique. This high sensitivity allows the quantification of cells labeled with iron oxides in very small volumes ($75 \mu\text{l}$). The EPR method also allows easy optimization of cell labeling protocols. Microscopy will of course remain essential to confirm that iron oxides are well localized in the cytosol and do not merely adhere to plasma membranes.

Although other techniques are also very sensitive (e.g. ICP-MS), EPR exhibits a major advantage, the intrinsic specificity for iron oxide compared with iron species contained in biofluids, which are not superparamagnetic. As a consequence, *ex vivo* measurements of tissue samples could be performed after MRI studies. Such experiments could be highly relevant for discriminating hypointense areas owing to artifacts (tissue interfaces, presence of air) and hypointense

regions owing to the labeled cells (13,21). Therefore, EPR could provide an important contribution to an unambiguous assessment of iron oxide content in tissues in MRI cell-tracking experiments.

4. EXPERIMENTAL

4.1. Cell Culture

Murine melanoma B16F10-luc were grown in Dulbecco's modified Eagle medium, with GlutaMAX™ I, 1000 mg l⁻¹ D-glucose, sodium pyruvate (Invitrogen, Merelbeke, Belgium), supplemented with 10% fetal bovine serum (Invitrogen, Merelbeke, Belgium) and 1% (v/v) penicillin–streptomycin (Invitrogen, Merelbeke, Belgium). Murine renal adenocarcinoma RENCA-luc were grown in RPMI-1640 medium with L-glutamine (Invitrogen, Merelbeke, Belgium), supplemented with additional L-glutamine 2 mM (Invitrogen, Merelbeke, Belgium), 10% (v/v) fetal bovine serum, nonessential amino acids 0.1 mM (Invitrogen, Merelbeke, Belgium), additional sodium pyruvate 1 mM (Invitrogen, Merelbeke, Belgium) and 1% (v/v) penicillin–streptomycin. Murine 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium, with L-glutamine, 4500 mg l⁻¹ D-glucose and 25 mM HEPES buffer, without sodium pyruvate (Invitrogen, Merelbeke, Belgium), supplemented with 10% (v/v) heat-inactivated newborn calf serum (Invitrogen, Merelbeke, Belgium), sodium pyruvate 1 mM and 1% (v/v) penicillin–streptomycin.

4.2. MIRB Localization After Cell Labeling

3T3, B16F10-luc and Renca-luc cells were seeded at low concentration on sterile glass coverslips. Cells were then incubated with Molday ion rhodamine B particles (Biopal, Worcester, USA) at 100 µg Fe/2 ml/9.6 cm² in medium overnight. These particles are especially designed for cell labeling experiments and were chosen because of their good uptake by cells and their lack of toxicity (14). After the incubation, cells were rinsed three times with phosphate-buffered saline (PBS; Lonza, Braine-l'Alleud, Belgium), fixed with paraformaldehyde 4% (Sigma-Aldrich, Bornem, Belgium), and washed three times with PBS containing 0.1% Triton for membrane permeabilization. Actin was stained using phalloidin-fluorescein isothiocyanate (FITC) (0.5 mg ml⁻¹ in methanol, diluted 1:250 in PBS; Sigma-Aldrich, Bornem, Belgium). After three new washes with PBS, nuclei were stained using Hoechst 33342 (0.02 g in 10 ml distilled water, diluted 1:10 000; Sigma-Aldrich, Bornem, Belgium). Coverslips were then mounted using Vectashield H1000 (Vector Labs, Brussels, Belgium) and slides were analyzed using a fluorescence microscope Zeiss Axio Imager equipped with an ApoTome module (pseudo-confocal).

4.3. Sample Preparation for Iron Quantification

For the experiment comparing the methods, 3T3 cells were seeded one day before the experiment (10 000 cell cm⁻²) in six-well plates (three wells per method; Sarsedt, Essen, Belgium). At day 0, the medium was removed and replaced by a mix solution containing full medium and MIRB particles (28.5 µg Fe/2 ml/9.6 cm²) for an overnight incubation. Cells were washed four times with PBS the next day, gently harvested with 1 ml of trypsin EDTA 0.05% (Invitrogen, Merelbeke, Belgium) and neutralized with 1 ml of full medium. Cells were centrifuged at 180g for 5 min and resuspended in 500 µl cold PBS in a 1.5 ml Eppendorf cup. A 10 µl

aliquot of the cell suspension was collected and mixed with 10 µl of trypan blue (Sigma-Aldrich, Bornem, Belgium) for cell counting using a hemocytometer. In the mean time, the cell suspension was centrifuged at 160g for 3 min. The 3T3 fibroblasts were eventually resuspended in 200 µl PBS for EPR analysis, in 200 µl HCl 5 M for Perls' reaction-based colorimetry, in 1 ml PBS for fluorimetry and 500 µl PBS for ICP-MS.

For the kinetics of MIRB uptake, Renca-luc cells (15 000 cells cm⁻²) and B16F10-luc (10000 cells cm⁻²) were seeded the day prior to the experiment in 60 mm tissue dishes (Sarsedt, Essen, Belgium). The next day, the medium was removed and cells were washed with PBS to remove traces of serum; then 4 ml of a final solution containing full or serum free medium and MIRB particles (80 µg Fe/4 ml/28 cm²) was added. Cells were collected at each time point using the same protocol as the methods comparison protocol except that the cells were finally resuspended in 300 µl of PBS. EPR measurements were done in triplicate.

4.3.1. Perls' reaction-based colorimetry

The sample resuspended in 200 µl HCl was digested for 24 h at 37 °C in closed Eppendorfs with regular agitation. After that the solution was mixed with 100 µl of distilled water containing 5% potassium ferrocyanide (Sigma-Aldrich, Bornem, Belgium), the Perls' reagent. The optical density at 700 nm (OD₇₀₀) was measured 15 min after addition of the Perls reagent in a 96-well plate (200 µl per well) using a Molecular Devices SpectraMax 190 microplate reader. Calibration curves were performed with different MIRB dilutions (from 20 to 0 µg Fe ml⁻¹) in HCl 5 M mixed with ferrocyanide 5% solution (ratio 2:1).

4.3.2. Electron paramagnetic resonance

Cells resuspended in PBS were drawn into a 75 µl glass capillary tubes. Samples were analyzed at room temperature using a Bruker EMX EPR spectrometer operating at 9 GHz (Bruker Biospin GmbH, Germany) with the typical parameters: 3 mT modulation amplitude, 10.11 mW power, 325.1 mT center field, and field 400 mT sweep width. Signal intensity was recorded using a double integration of the iron oxides EPR spectra. Calibration curves were realized in the same conditions with MIRB solutions at different concentrations (from 20 to 0 µg Fe ml⁻¹).

4.3.3. Fluorimetry

Samples in 1 ml PBS were analyzed using PerkinElmer LS 55 with quartz cuvettes. MIRB emission spectra were recorded with the following parameters: 555 nm excitation, maximum emission at 578 nm and 9 nm slit. MIRB calibration curves (from 20 to 0 µg Fe ml⁻¹) were realized in the same conditions.

4.3.4. ICP-MS

The samples were analyzed by inductively coupled argon plasma with mass spectrometry using an Agilent 7500ce instrument. Briefly, samples (50 µl) were diluted quantitatively (1:100) with a HNO₃ 1%, HCl 0.5% solution containing all internal standards (Sc, Ge, Rh and Ir). Fe was quantified using

helium mode (collision cell) and its isotope at m/z 56 with Ge (at m/z 74) as internal standard.

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II. Multimodal cell tracking of a spontaneous metastasis model: comparison between MRI, electron paramagnetic resonance and bioluminescence

Pierre Danhier, Géraldine De Preter, Julie Magat, Quentin Godechal, Paolo E. Porporato, Bénédicte F. Jordan, Olivier Feron, Pierre Sonveaux and Bernard Gallez

Thanks to the use of fluorescence microscopy and EPR for assessing the (U)SPIO localization as well as the labeling efficiency after passive incubation of cells with iron oxides, it was found that luciferase-expressing renal adenocarcinoma RENCA-luc cells efficiently internalized Molday Ion Rhodamine B (MIRB) USPIO. We next assessed the fate of MIRB-labeled metastatic RENCA-luc cancer cells at distant sites using a multimodal approach combining MRI and BLI. As the previous study highlighted that EPR was specific for iron oxide content, *ex vivo* EPR measurements of mouse tissues were performed to validate MRI cell tracking experiments.

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Multimodal cell tracking of a spontaneous metastasis model: comparison between MRI, electron paramagnetic resonance and bioluminescence

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MRI cell tracking is a promising technique for tracking various cell types in living animals. Usually, cells are incubated with iron oxides so that the particles are taken up before the cells are injected *in vivo*. In the present study, we aimed to monitor migration of luciferase-expressing mouse renal cancer cells (RENCA-luc) after intrarenal or intrasplenic injection. These cells were labelled using Molday Ion Rhodamine B (MIRB) fluorescent superparamagnetic iron oxide particles. Their fate after injection was first assessed using *ex vivo* X-band electron paramagnetic resonance (EPR) spectroscopy. This biodistribution study showed that RENCA-luc cells quickly colonized the lungs and the liver after intrarenal and intrasplenic injection, respectively. Bioluminescence imaging (BLI) studies confirmed that this cell line preferentially metastasized to these organs. Early tracking of labelled RENCA-luc cells in the liver using high-field MRI (11.7 T) was not feasible because of a lack of sensitivity. MRI of MIRB-labelled RENCA-luc cells after injection in the left kidney was then performed. T_2 - and T_2^* -weighted images showed that the labelled cells induced hypointense signals at the injection site. Nevertheless, the hypointense regions tended to disappear after several days, mainly owing to dilution of the MIRB iron oxides with cell proliferation. In conclusion, EPR is well adapted to *ex vivo* analysis of tissues after cell tracking experiments and allows short-term monitoring of metastasizing cells. MRI is a suitable tool for checking labelled cells at their injection site, but dilution of the iron oxides owing to cell division remains a major limitation. BLI remains the most suitable technique for long-term monitoring of metastatic cells. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: electron paramagnetic resonance; magnetic resonance imaging; bioluminescence; cell tracking; metastasis; cancer

1. INTRODUCTION

Cell tracking can be defined as the noninvasive monitoring of cells *in vivo* after implantation. While precise localization of the injection site is mainly relevant for cell-based therapies [e.g. cardiac injuries (1), cerebral strokes (2), cartilage repair (3)], the ability to follow up cell migration is also critical in understanding complex multi-step events such as cancer metastasis. Two approaches currently exist: direct labelling involves incorporation of a contrast agent within the cell, whereas indirect strategies involve reporter genes. Different imaging modalities [MRI (4), optical imaging (5) and PET (6)] can be used for cell tracking and combine different advantages and drawbacks (7). MRI, for example, has excellent spatial resolution, and many contrast agents are available, such as ¹⁹fluorine (8), Gd-based contrast agents (9) and iron oxides (10). Superparamagnetic particles of iron oxides (SPIO) are the most widely used contrast agents for cell labelling studies since they induce strong hypointense signals on T_2 - and T_2^* -weighted images and are biocompatible (10). MRI cell labelling has been performed on several cell types such as stem cells (11,12), macrophages (13) and cancer cells (14). Nevertheless, MRI has poor sensitivity

[although single-cell tracking has been reported (15)] and cell proliferation can lead to contrast agent dilution (16). On the other hand, bioluminescence imaging (BLI) of luciferase-expressing cells is a typical indirect cell tracking strategy with high sensitivity. Since BLI is a reporter gene-based method, only living cells that express the luciferase gene will emit luminescence. The reaction is based on the oxidation of D-luciferin by the luciferase, which results in the emission of photons. Nevertheless, a major limitation is the lack of spatial resolution (17). Another disadvantage is the low penetration of photons through tissues, which limits this method to small animal studies (18).

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Metastasis, the first cause of cancer-related death (19), comprises multiple steps including intravasation of cancer cells, their circulation in the blood stream, their extravasation into tissues of distant organs and colonization (19). In basic research, one can distinguish experimental metastasis (i.e. following injection of cancer cells into the blood stream) from spontaneous metastasis (i.e. arising after orthotopic injection of cancer cells), the latter aiming to reflect all the steps in the metastatic cascade (20). Metastasis is mainly assessed in fundamental research by inspection of macroscopic lesions several days after the injection of cancer cells (21). Although *in vivo* microscopy imaging modalities exist (22), SPIO-based cell tracking could be a tool for noninvasively monitoring the fate of cancer cells after implantation.

In the present study, we aimed to use a multimodal approach to track metastatic cells. We used BLI as a method for assessing the long-term development of viable cells, and compared it with MRI cell tracking, known to offer excellent spatial resolution (23,24). We also used electron paramagnetic resonance (EPR) to validate SPIO-based cell tracking for monitoring metastasis patterns of a murine spontaneous model of metastasis. Indeed, *ex vivo* EPR spectroscopy is a sensitive and suitable technique for quantifying iron oxide nanoparticles in organs (25–28). We then addressed the question of whether MRI could be useful in detecting distant metastasis. Therefore, we used a luciferase-positive murine renal adenocarcinoma cell line (RENCA-luc) known to form spontaneous lung metastases after orthotopic injection (29). This cell line is also described as colonizing the liver after intrasplenic injection (experimental metastasis) (30).

METHODS

2.1. Cell Culture

Murine renal adenocarcinoma RENCA cells (ATCC, Molsheim, France) were grown in RPMI-1640 medium with L-glutamine (Invitrogen, Merelbeke, Belgium), supplemented with additional L-glutamine 2 mM (Invitrogen), 10% (v/v) foetal bovine serum, nonessential amino acids 0.1 mM (Invitrogen), additional sodium pyruvate 1 mM (Invitrogen) and 1% (v/v) penicillin–streptomycin (Invitrogen).

Luciferase-expressing RENCA cells (RENCA-luc) were generated by lentiviral infection with pLenti PGK V5-LUC Neo transfer vector (addgene plasmid 21471) (31). This plasmid was co-transfected in 293FT cells with pMDLg/pRRE, pRSV-Rev and pMD2.VSVG in order to produce a high-titre lentiviral vector. Infected cells were selected for geneticin resistance and then tested for luciferase activity.

2.2. Cell Labelling and Assay for Dilution of SPIO Particles

Two days prior to the experiment, RENCA-luc cells were seeded in six-well plates (for EPR quantification) or 24-well plates (for fluorescence microscopy) at 10^4 cells/cm². On the next day, the cells were incubated overnight with Molday Ion Rhodamine B (MIRB) fluorescent SPIO (Biopal, Worcester, USA) in a serum-free medium at a concentration of 40 µg Fe ml⁻¹. These conditions have been shown to be optimal for obtaining high yields of intracellular MIRB incorporation (32). After labelling, the incubation medium was discarded. Cells were washed with PBS and replenished with full medium. Cells were collected after labelling (day 0), and on days 1 and 2 post-labelling for intracellular iron oxide quantification, as previously described (32). Briefly, RENCA-luc cells were washed three times with PBS and

harvested using trypsin. After trypsin neutralization using medium-containing serum, samples were centrifuged (180 g for 5 min). Supernatants were carefully discarded and the cells were resuspended in PBS. At this step, cells were counted using a haemocytometer and recentrifuged (180 g for 3 min). The cell suspension was finally resuspended in 1 ml PBS for EPR analysis. Aqueous samples were analysed at room temperature using a Bruker EMX EPR spectrometer operating at 9 GHz (Bruker Biospin GmbH, Germany) with the following parameters: microwave power, 2.02 mW; centre field, 3150 G; field sweep width, 4000 G; modulation amplitude, 30 G; time constant, 20.48 ms; conversion time, 20.48 ms; modulation field, 100 kHz. Calibration curves were recorded under the same conditions with MIRB suspensions at different concentrations (from 40 to 0 µg Fe ml⁻¹). Measurements were performed at room temperature. Fluorescence microscopy was meanwhile performed on days 0, 1 and 2 post-labelling using an AxioVert S100 microscope (Zeiss).

2.3. Ethical Statement

All *in vivo* experiments were performed in compliance with guidelines set by national regulations and were approved by the ethical committee for animal care of the Université catholique de Louvain.

2.4. Animal Experiments

RENCA-luc cells were seeded in T25 flasks at a cell density of 3×10^4 cells cm⁻². On the day prior to injection, the cells were incubated with 5 ml of serum-free medium containing MIRB nanoparticles (40 µg Fe ml⁻¹). The next day, MIRB-labelled RENCA-luc (or control cells) were collected using the same protocol as for the MIRB dilution assay and kept on ice in a PBS solution. Aliquots of samples were used for intracellular iron oxide quantification using EPR as previously described (32). Then, 8-week-old male Balb/cJrJ mice (Janvier) were anaesthetized with ketamine (80 mg kg⁻¹)/xylazine (8 mg kg⁻¹) and their left flanks were depilated. A 1 cm dorsal skin incision was made and the left kidney or the spleen was exposed. RENCA-luc cells (10^5 cells for *ex vivo* EPR and BLI or 3×10^5 cells for MRI) or MIRB (1 µg Fe for *ex vivo* EPR, 3 µg Fe for MRI) were then injected into the renal cortex or into the spleen using a syringe equipped with a 26 s gauge needle (Hamilton, Switzerland). For the *in vivo* MRI experiments, animals were anaesthetized by inhalation of isoflurane (Forene, Abbot, UK) mixed with 21% oxygen (air) in a continuous flow (1.5 L h⁻¹). The temperature of the mice was maintained at 37.0 ± 1.0 °C using a water blanket connected to a circulating water bath, and respiration was monitored.

2.5. Biodistribution of Iron Oxide Nanoparticles Using Ex Vivo EPR

Balb/cJrJ mice injected (in the spleen or in the left kidney) with MIRB-labelled cells or MIRB nanoparticles were sacrificed at day 1, 3 or 8 post-injection. The liver, spleen, lungs and left kidney were harvested, freeze-dried, and crushed into a fine powder. Then, all samples were weighed and the iron oxide content was determined using EPR spectroscopy (26,27). The EPR parameters were as follows: microwave power, 5.05 mW; centre field, 3150 G; field sweep width, 5000 G; modulation amplitude, 30 G; time constant, 20.48 ms; conversion time, 20.48 ms; modulation field, 100 kHz. Measurements were performed at

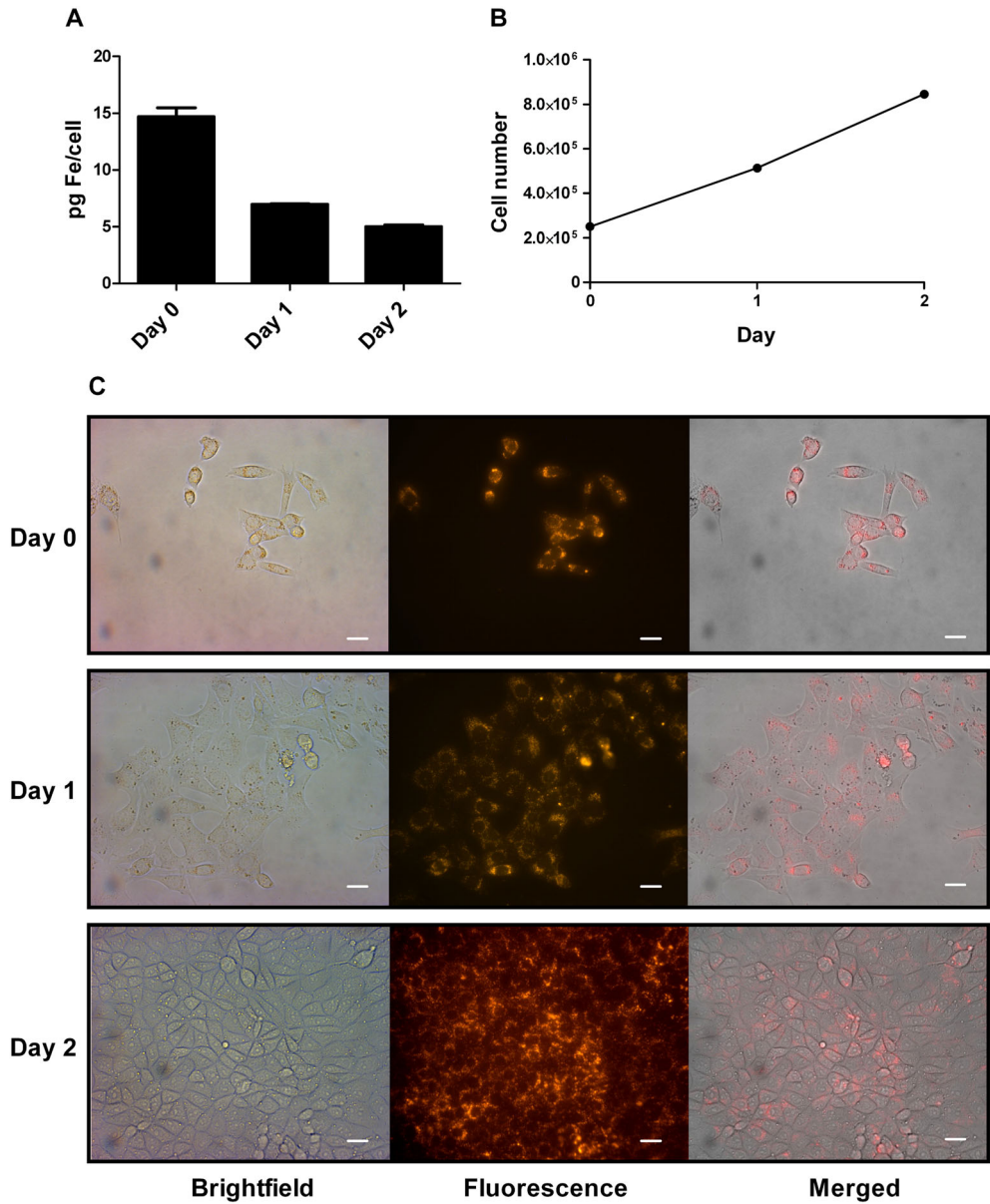


Figure 1. Dilution of intracellular Molday Ion Rhodamine B (MIRB) particles with cell proliferation. (A) The intracellular iron oxide content (pg Fe per cell) decreased with time ($n = 4$) in MIRB-labelled cells. Meanwhile, the cell number increased (B). (C) Bright field, fluorescence and merged microscopy pictures of RENCA-luc cells containing red fluorescent nanoparticles are shown at different times after labelling. Scale bars in white represent 20 μm. Data are represented as means ± SEM.

room temperature. The iron oxide content in each freeze-dried organ was quantified by comparison with a calibration curve of freeze-dried MIRB standards.

2.6. Magnetic Resonance Imaging

In vivo MR experiments were performed with an 11.7T system (Bruker, Biospec) with a volume transmit coil (diameter = 72 mm) coupled with a receive coil phase array with four elements. All acquisitions were triggered by mouse respiration. T_2 -weighted (T_2w) images were acquired using a multi-slice multi-echo sequence with the following parameters: repetition time, 1200 ms; echo time, 15 ms; flip angle, 112.6°; matrix, 384 × 196; slice thickness, 1 mm; number of averages, 3; total acquisition time, 8 min 52 s. T_2^* -weighted (T_2^*w) images were acquired using a multi

gradient echo sequence with the following parameters: repetition time, 1200 ms; echo time, 3.5 ms; excitation pulse angle, 30°; matrix, 256 × 256; slice thickness, 1 mm; number of averages, 3; total acquisition time, 11 min 31 s. The signal void was calculated using the following $(S_{hk} - S_{roi})/S_{bg}$, where S_{hk} is the signal intensity of the healthy kidney, S_{roi} the signal intensity of the region of interest and S_{bg} the signal intensity of the background (14).

2.7. Bioluminescence Imaging

Balb/cJrj mice injected (in the spleen or in the left kidney) with 10⁵ RENCA-luc cells were imaged on days 1, 8 and 15 post-injection. To parallel the experiments involving MIRB cell labelling, cells were incubated on the day prior to the experiment, in serum-free medium. Mice were anaesthetized

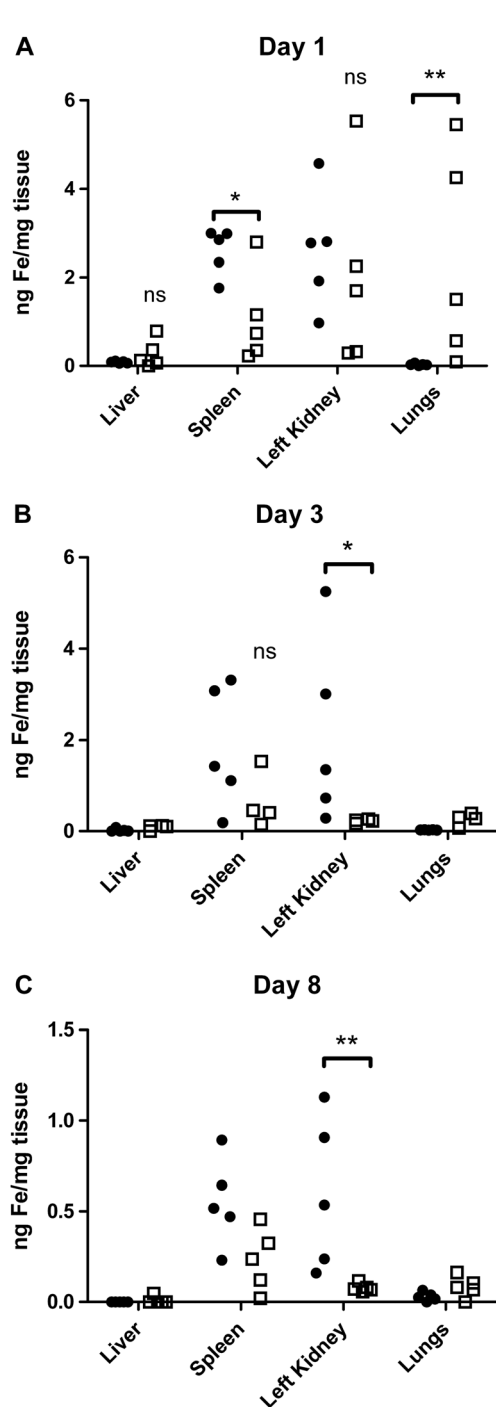


Figure 2. Biodistribution of MIRB iron oxides following intrarenal injection of MIRB-labelled RENCA-luc cells (□) or free MIRB particles (●). One day post-injection (A), iron oxides accumulated preferentially in the lungs of treated mice (RENCA-luc+MIRB), whereas free MIRB were found in the spleens of control mice. On days 3 (B) and 8 (C), iron oxide levels dropped for each group in all analysed organs. Statistical differences in iron oxide content in the lungs between mice receiving labelled cells and mice injected with free particles were no longer found. With regard to the injection site, the decrease in iron oxide content was more pronounced in the kidney of treated mice than mice receiving free MIRB particles. Each individual measurement is represented ($n=5$, except for RENCA-luc+MIRB group on day 3, where $n=4$); ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

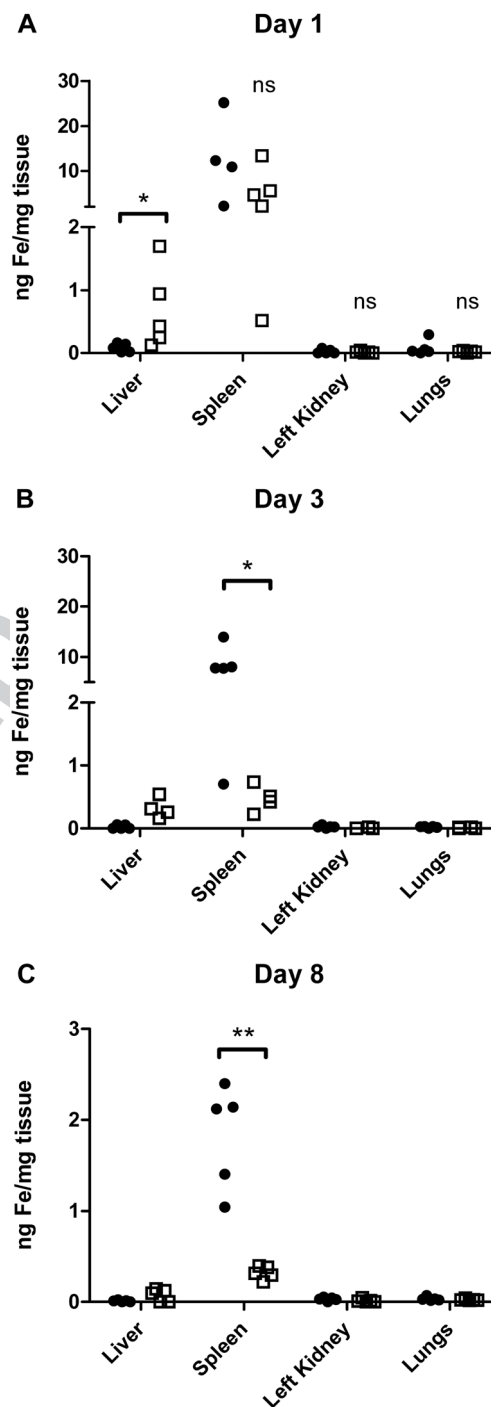


Figure 3. Biodistribution of MIRB iron oxides following intrasplenic injection of MIRB-labelled RENCA-luc cells (□) or free MIRB particles (●). Iron oxides were found to accumulate in the livers of mice injected with labelled cells compared with the free MIRB group one day post-injection (A); no significant amounts of MIRB particles were detectable in the lungs or the kidneys for either group. The MIRB content at the injection site (spleen) dropped on day 3 (B) and day 8 post-injection (C), especially for the RENCA-luc+MIRB group. No significant differences in iron oxide content between mice injected with MIRB-labelled cells and mice injected with free particles were observed in the livers of injected mice on days 3 and 8 post-injection. Each individual measurement is represented ($n=5$ except for spleens of MIRB group on day 1, where $n=4$); ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

with ketamine (80 mg kg^{-1})/xylazine (8 mg kg^{-1}) and depilated. Luciferin (150 mg kg^{-1} body weight) was injected intraperitoneally and BLI images were acquired 15 min after injection using an IVIS50 imaging system (Xenogen) and a 16-bit digitizer. Acquisition time was set at 1 min for all animals except for mice imaged on day 15, for which the exposure time was shortened to 20 s. At the end of the experiment (day 15), the mice were sacrificed and pictures of the organs colonized with RENCA-luc cells were taken.

2.8. Statistical Analyses

All results are represented as means $\pm$ standard error of the mean (SEM) except for the *ex vivo* EPR biodistribution study, where individual values are plotted. Mann–Whitney tests were conducted for the *ex vivo* EPR biodistribution study. MRI results were analysed using one-way ANOVA followed by Dunnett's multiple comparison test. Comparisons between EPR iron oxide quantification results on days 1, 3 and 8 were analysed using a Kruskal–Wallis test followed by a Dunn's multiple comparison test. Values of $p < 0.05$ were considered to be statistically significant.

3. RESULTS

3.1. Intracellular Iron Oxide Content is Diluted with Cell Proliferation

Intracellular iron oxide content was first measured at various times after cell labelling in order to study the persistence of iron

oxide nanoparticles inside the cell. As shown in Fig. 1(A), the intracellular MIRB content of labelled cells decreased over time ($14.71 \pm 0.78 \text{ pg Fe per cell}$ on day 0, 6.95 ± 0.06 on day 1, and 5.01 ± 0.15 on day 2 post-labelling). In parallel, the cell number increased [Fig. 1(B), 2.5×10^5 on day 0, 5.13×10^5 on day 1, 8.46×10^5 on day 2]. On the fluorescence images (Fig. 1C), the remaining red fluorescent MIRB particles were still located within the cell cytoplasm 2 days post-labelling.

3.2. Ex Vivo EPR Allows Short-Term Cell Tracking of RENCA-luc Tumour Cells

Next, we monitored the fate of MIRB-labelled RENCA-luc cells [$10 \text{ pg Fe per cell}$ assessed using EPR (32)] injected into the spleen or into the kidney of the mice. When MIRB-labelled cells were injected into the kidney, an accumulation of iron oxide particles was observed in the lungs of mice injected with the labelled cells one day post-injection. Interestingly, this increase was not observed in the control group, that is, for MIRB alone (Fig. 2A, $2.38 \pm 1.06 \text{ ng Fe mg}^{-1}$ tissue for the RENCA-luc + MIRB group vs $0.03 \pm 0.01 \text{ ng Fe mg}^{-1}$ tissue for the MIRB group). After intrarenal injection, iron oxides alone were also found to accumulate preferentially in the spleen (Fig. 2A, $1.05 \pm 0.46 \text{ ng Fe mg}^{-1}$ tissue for the RENCA-luc + MIRB group vs $2.59 \pm 0.23 \text{ ng Fe mg}^{-1}$ tissue for the MIRB group). No difference in MIRB content was observed in the liver regardless of the group of mice (Fig. 2A, $0.27 \pm 0.14 \text{ ng Fe mg}^{-1}$ tissue for the RENCA-luc + MIRB group vs $0.08 \pm 0.01 \text{ ng Fe mg}^{-1}$ tissue for the MIRB group). For mice having

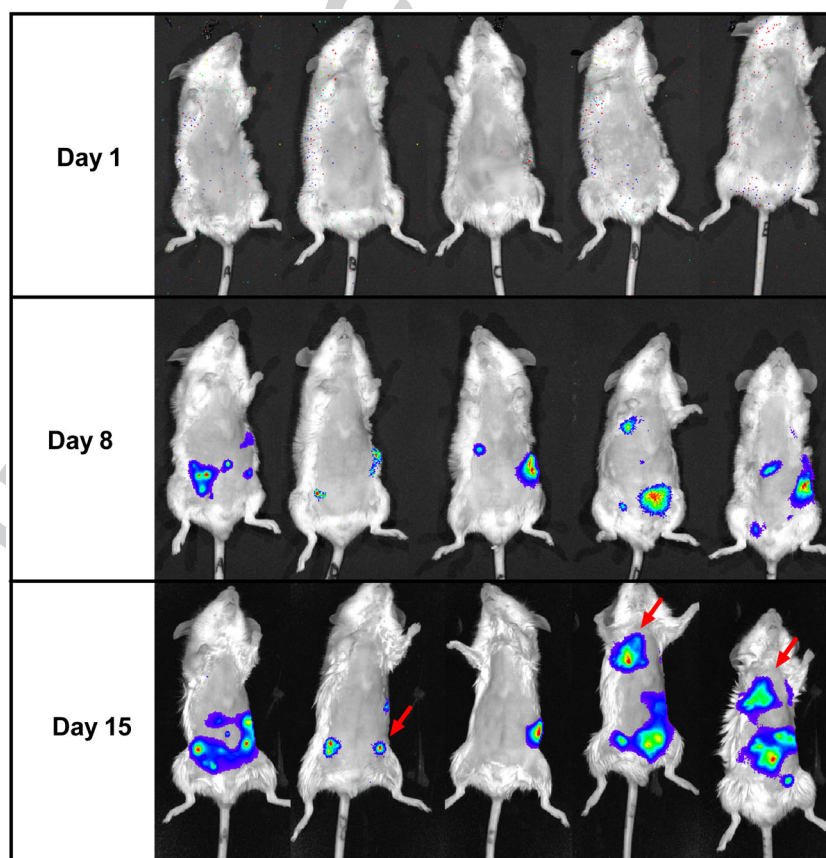


Figure 4. Bioluminescence imaging (BLI) of mice injected in the left kidney with RENCA-luc cells. BLI performed on days 1, 8 and 15 post-injection showed that RENCA-luc cells metastasized to the lungs and lymph nodes (red arrow). It is noteworthy that no visible lesion was apparent on day 1. The same mice ($n = 5$) were imaged in the course of the experiment and were pictured individually.

received labelled cells in the spleen (Fig. 3), a higher iron oxide accumulation one day after injection was observed in the liver in the RENCA-luc+MIRB group compared with the control group (Fig. 3A, $0.69 \pm 0.29 \text{ ng Fe mg}^{-1}$ tissue for the RENCA-luc+MIRB group vs $0.08 \pm 0.03 \text{ ng Fe mg}^{-1}$ tissue for the MIRB group). These differences were no longer observed on days 3 and 8 post-injection (Figs 2B, 2C, 3B and 3C). Surprisingly, iron oxide levels dropped after 3 days in organs injected with labelled cells. These decreases in iron oxide content were more pronounced for the RENCA-luc+MIRB groups than the free MIRB groups (Fig. 2B and C for intrarenal injection; Fig. 3B and 3C for intrasplenic injection).

3.3. Bioluminescence Imaging Confirms That RENCA-luc Cells Metastasize Preferentially to the Lungs and the Liver

In order to confirm that iron oxide nanoparticle accumulation in the lungs (after intrarenal injection) or in the liver (after intrasplenic injection) could reflect cancer cell dissemination from the injection site to these organs, we investigated the metastatic pattern of RENCA-luc cells after intrarenal (spontaneous metastasis) or intrasplenic (experimental metastasis) injection in mice using BLI on days 1, 8 and 15 post-injection. When cells were injected into the left kidney (Fig. 4), luminescent positive spots were observed in the lungs and lymph nodes several days after

injection (days 8 and 15). Mice receiving RENCA-luc cells in the spleen (Fig. 5) displayed positive spots in the liver and in the spleen (days 8 and 15). No positive spot was visible on day 1 after intrasplenic injection except at the injection site. As the mice were lying in a supine position, no signal arising from the kidneys was detected one day after intrarenal injection (Fig. 4). All BLI-positive organs were imaged (Fig. 6) after the experiment to confirm that RENCA-luc cells colonized the lungs (intrarenal injection) and the liver (intrasplenic injection).

3.4. MRI is not Sensitive Enough to Detect Early Metastatic Labelled RENCA-luc Cancer Cells but Allows Visualization of Late Metastasis

We next tested whether liver metastasis could be detected using MRI cell tracking after intrasplenic injection of MIRB-labelled RENCA-luc cells. As shown in Fig. 7 (T_2w and T_2^*w images), it was not possible to distinguish any hypointense region owing to labelled cells from the surrounding liver parenchyma. Nevertheless, metastases were visible on day 17 post-injection as hyperintense lesions in the liver, indicating that RENCA-luc cells had successfully colonized this organ. As already shown in Fig. 6, these liver macrometastases could be detected visually after sacrifice. Spontaneous lung metastasis after intrarenal injection of

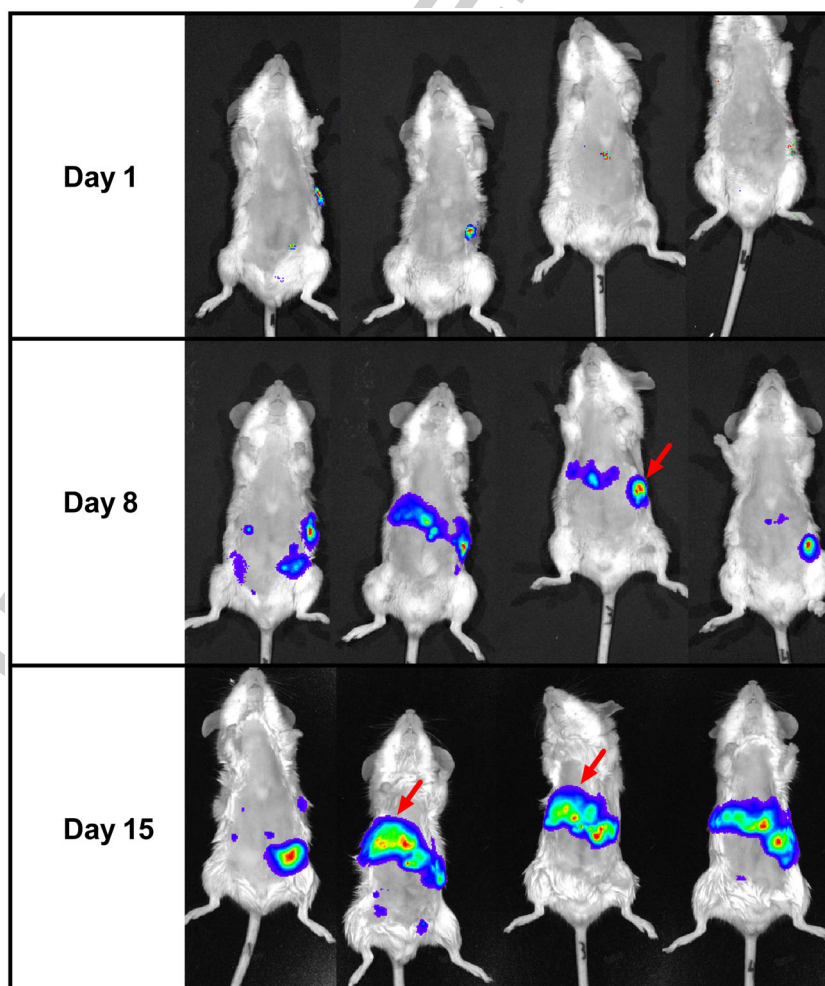


Figure 5. Bioluminescence imaging of mice injected in the spleen with RENCA-luc cells. BLI performed on days 1, 8 and 15 post-injection showed that RENCA-luc cells metastasized to the liver (red arrow) after intrasplenic injection. It is noteworthy that no visible lesion was apparent on day 1 (except for the injection site). The same mice ($n = 4$) were imaged in the course of the experiment and are pictured individually.

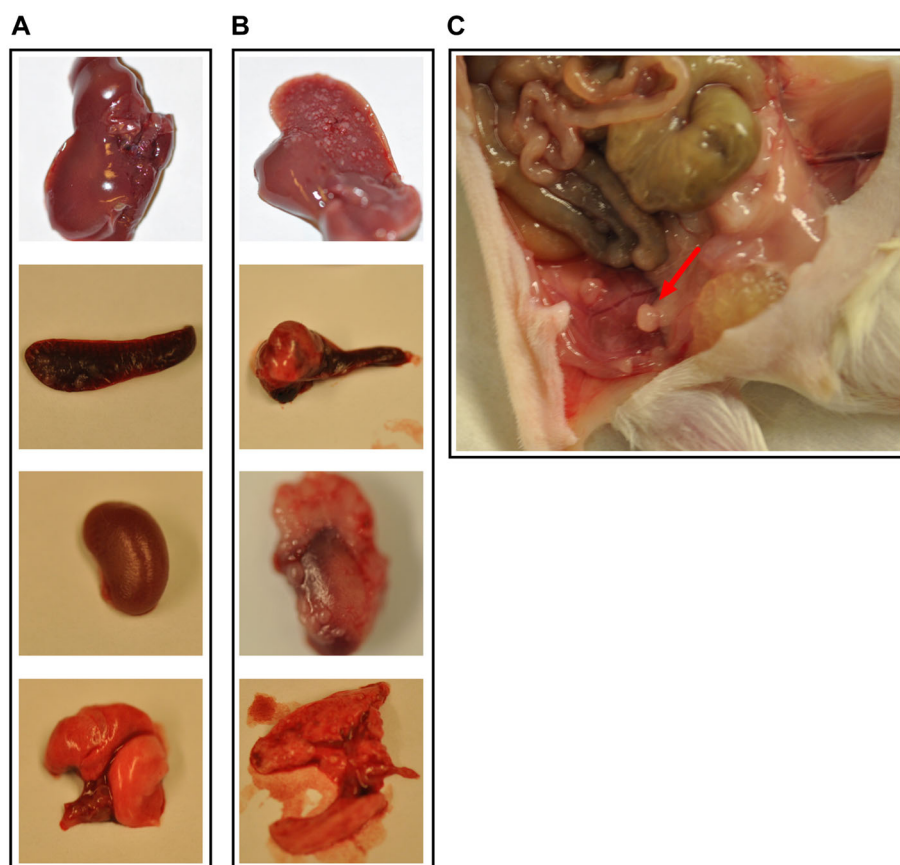


Figure 6. *Ex vivo* confirmation of the presence of RENCA-luc cells after BLI experiments. (A) Representative pictures of BLI-negative organs (B). Pictures of BLI-positive organs colonized by RENCA-luc cells. (C) Picture of a BLI-positive metastasis (red arrow).

MIRB-labelled RENCA-luc could not be visualized with MRI, as lungs appear as very hypointense spots on conventional T_2 - and T_2^* w images (33,34).

3.5. The Clearance of Iron Oxide Particles from the Tumour Cells Restricts SPIO-Based MRI Cell Tracking to Short-Term Studies

EPR results showed a rapid loss of iron oxides in organs injected with labelled cells, but on the other hand a long-lasting presence of iron oxides in organs receiving only MIRB particles (Figs 2 and 3). The fate of MIRB-labelled RENCA-luc cells implanted in the left kidney was further monitored with MRI. Hypointense regions owing to the presence of MIRB nanoparticles were observed at the injection sites on day 0 (Fig. 8). Nevertheless, the signal void decreased quickly over time on both the T_2 w and T_2^* w images. Finally, lesions appeared isointense on day 8 post-injection (Fig. 8B; T_2 signal void = 12.93 ± 3.64 on day 0 vs 0.45 ± 2.83 on day 8; Fig. 8C, T_2^* signal void = 20.00 ± 2.67 on day 0 vs -0.15 ± 3.22 on day 8). This contrast decay is in line with iron oxide quantification by *ex vivo* EPR. In Fig. 8(D), the iron oxide content assessed by *ex vivo* EPR decreased with time, and SPIO were totally cleared on day 8. Surprisingly, a mouse displayed a significant haemorrhage in the kidney (visualized in a different slice than the isointense signal arising from the tumour area) on day 8 (Fig. 8E), appearing also as confounding hypointense regions on T_2 -, T_2^* -weighted images. On T_2 w images, signal loss following injection of MIRB particles (Fig. 9) had significantly decreased on day 8, but a signal loss was still observable (Fig. 9B;

T_2 signal void = 8.64 ± 2.24 on day 0 vs 2.04 ± 1.66 on day 8). On the other hand, signal voids remained unchanged on T_2^* w images (Fig. 9C; T_2^* signal void = 20.78 ± 9.82 on day 0 vs 8.35 ± 3.40 on day 8). Iron oxide quantification by *ex vivo* EPR showed a loss of MIRB with time, but significant levels of SPIO were still detected on day 8 (Fig. 9D). As a supplementary control, PBS injections were also performed: hypointense areas were observed on day 0 (T_2 w and T_2^* w images) but disappeared quickly in the following days (data not shown).

4. DISCUSSION

In this study, we observed a rapid dilution of the intracellular iron oxide content after labelling owing to cell proliferation. It is noteworthy that SPIO remained in the intracellular compartment. This feature has already been reported in several studies and is a major drawback for long-term direct cell tracking (16,35). The use of MIRB in cell tracking studies has been reported to be nontoxic for labelled cells (36). Conversely, we also demonstrated that *ex vivo* EPR spectroscopy (9 GHz) could be a sensitive tool for monitoring the fate of SPIO-labelled cells after injection. Although *in vivo* EPR (1.2 GHz) has been used in previous studies for iron oxide quantification (26), this technique could not be implemented in the present study because (1) the sensitivity is dramatically decreased at lower frequencies and (2) commonly used surface coils are incompatible with measurements in deep organs such as the kidneys, the liver or the lungs. Based on the biodistribution study, it was shown that RENCA-luc tumour cells

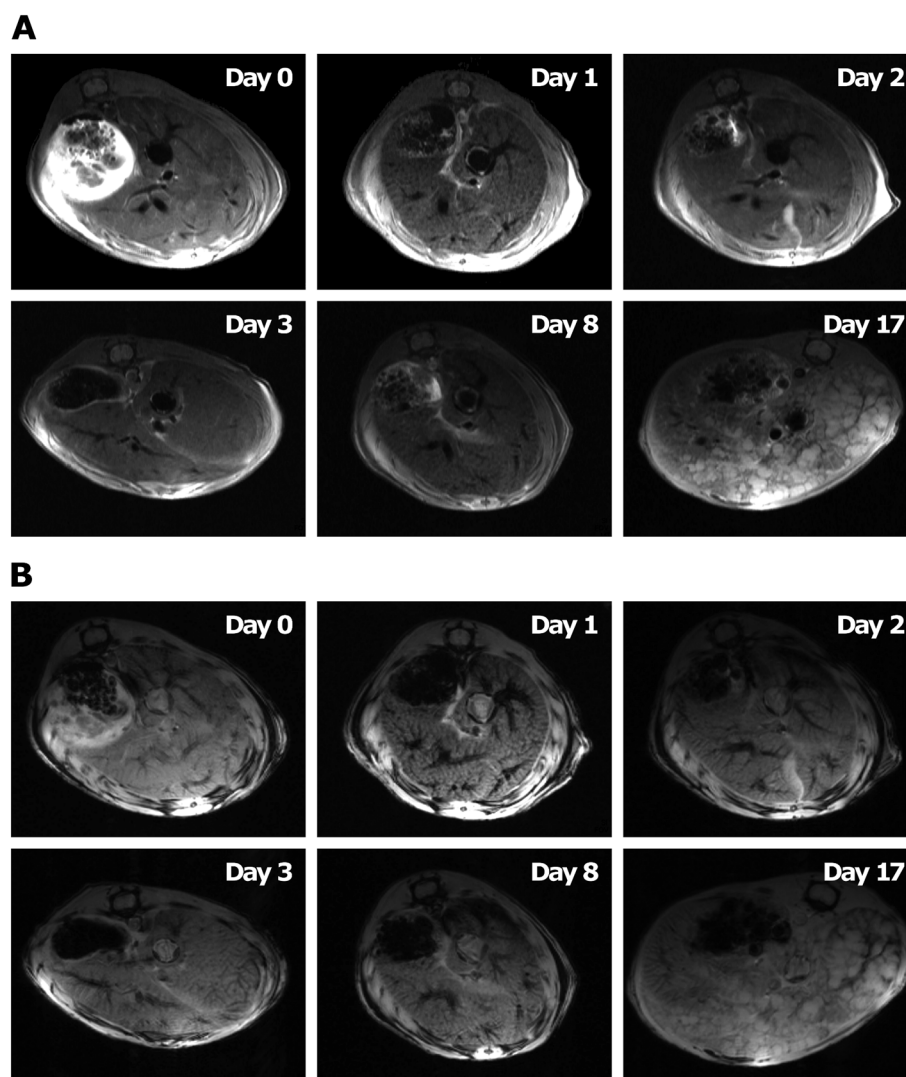


Figure 7. MR assessment of the fate of MIRB-labelled RENCA-luc cells after intrasplenic injection [T_2 -weighted (T_2w) and T_2^* w]. Representative axial T_2w (A) and T_2^*w (B) images of the liver on days 0, 1, 2, 3, 8 and 17 after injection of 3×10^5 labelled RENCA-luc cells. No MIRB-labelled cells were detectable on either T_2w or T_2^*w images. Hyperintense lesions owing to liver metastasis appeared at later times (day 17; $n=3$).

could migrate quickly to the liver after intrasplenic injection or to the lungs after orthotopic injection in the left kidney. This last observation challenges the idea that the RENCA-luc cell line is a spontaneous model of metastasis (29). It is indeed more likely that cells are directly transported in the renal blood flow after injection, as labelled cells were found in the lungs one day after intrarenal implantation. The metastatic pattern of this cell line was confirmed with bioluminescence imaging. EPR spectroscopy was thus able to detect cells just one day following injection. We therefore think that this method could be an interesting tool for cell tracking studies (e.g. to study migration of transplanted cells (2,37)). It would have advantages when considering organs that are difficult to image using conventional MRI, for example, the lungs (33,34). Finally, the observed drop in the iron oxide content (especially for labelled cells) on days 3 and 8 in organs injected with free SPIO or SPIO-labelled cells is surprising, although MR images confirmed a loss of iron oxide content with time. This is most likely explained by SPIO metabolism and uptake of released iron oxide particles by macrophages (38,39). Iron oxide quantification results with *ex vivo* EPR are in line with MRI data on the kidney. In fact, we found that iron oxide clearance from the

kidney was faster in the MIRB-labelled cell group after intrarenal injection, whereas significant levels of SPIO were still detectable 8 days after injection of free MIRB. This could be visualized on MR images, where the isointense signal arising from the tumour was observed on day 8 after injection of labelled cells. In contrast, T_2^* negative contrast owing to iron oxides was still present 8 days after injection of free particles. This good agreement between MRI and quantitative *ex vivo* EPR shows that the methods can be complementary for cell tracking applications.

Based on our observations at 11.7T, it was not possible to visualize MIRB-labelled cells in the liver after intrasplenic injection. However, successful cell tracking of hepatocytes after intrasplenic injection has been reported (40). The main difference with the present study is that the hepatocytes were labelled with micron-sized iron oxides (diameter of the MPIO, 1.63 μm). These particles contain many more superparamagnetic cores than the SPIO used in the present study (diameter of the MIRB, 35 nm) so that a higher intracellular iron oxide content can be reached. Secondly, MPIO are characterized by a much higher transverse relaxivity than SPIO (41). However, we were

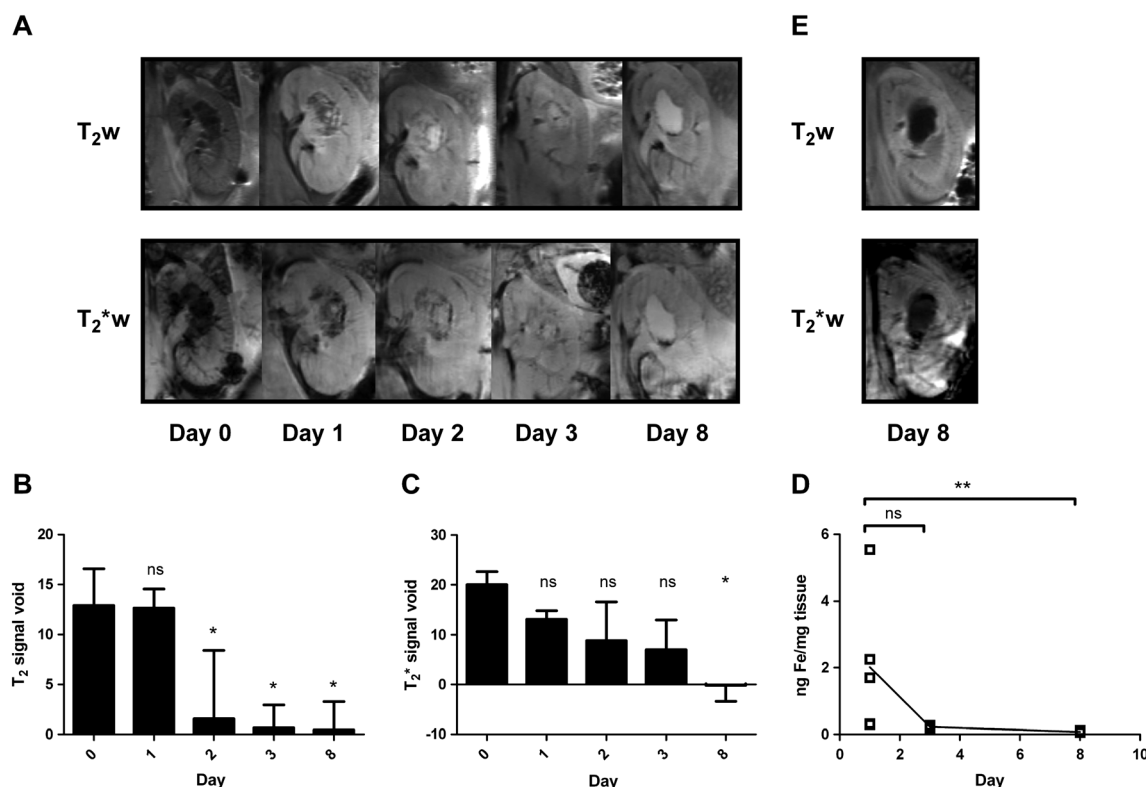


Figure 8. MR monitoring of MIRB-labelled RENCA-luc after injection in the left kidney. (A) Representative coronal images (T_2w and T_2^*w) of the left kidney on days 0, 1, 2, 3 and 8 after injection of 3×10^5 labelled RENCA cells. Signal voids on T_2w (B) and T_2^*w (C) decreased with time. The lesions appeared isointense on day 8 post-injection ($n = 3$). (D) *Ex vivo* EPR showed that iron oxides were quickly eliminated after injection of MIRB-labelled cells (10^5) and were cleared from the kidney on day 8. (E) MR images (T_2w and T_2^*w) of a kidney on day 8 post-injection displaying an important haemorrhage (hypointense area). Data are represented as means $\pm$ SEM except for (D), where each individual measurement is represented; medians are crossed by the connecting curve. ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

able to visualise MIRB-labelled RENCA-luc cells in the kidney after injection. Signal voids decreased with time owing to tumour growth, SPIO degradation and potential cell death, as well as the presence of macrophages known to phagocytose released iron oxide particles (39). These observations confirm that SPIO-based cell labelling is probably not a valid method for long-term *in vivo* monitoring of cancer cells. Moreover, the presence of haemorrhages owing to tumour growth (42) or injection could be an important confounding factor (43).

We also found using BLI that SPIO-labelled cells detected using *ex vivo* EPR in the liver or in the lungs established metastases several days after injection. The combination of magnetic labelling and BLI allowed early events in the metastasis cascade, that is, the entrapment of circulating cells in distant organs and subsequent colonization of these organs by cancer cells, to be monitored. As mentioned, dilution of intracellular SPIO owing to cell division is an important limitation of SPIO-based cell tracking. BLI is based on the use of an optical reporter gene and signal intensity is preserved upon cell division. Therefore, BLI appears more convenient for long-term monitoring of the fate of highly proliferative cells such as cancer cells. Nevertheless, light scattering by tissues affects the resolution of BLI images (44). These issues could be solved using tomographic reconstruction techniques (44,45). Moreover, BLI is able to distinguish living cells from dead cells, whereas SPIO-based cell tracking is specific to the iron oxide content. When SPIO-labelled cells die, their iron oxide content is released, but is still detectable using MRI/EPR. In addition,

released particles can be taken up by macrophages and data interpretation might become problematic (39). On the other hand, the BLI signal is lost when luciferase-expressing cells die, so that interpretation of BLI results is straightforward (46). As shown on T_2w liver images on day 17, MRI allowed an accurate assessment of the size and number of macrometastases.

To conclude, we have validated EPR spectroscopy as a promising tool for assessing the fate of cells labelled with iron oxides. Although animal sacrifice is required, high sensitivity combined with the absence of an endogenous signal (47) offers prospects for cell tracking applications. Dilution of iron oxides with cell proliferation limits drastically the time period during which cells can be monitored using MRI cell tracking. Since SPIO particles are negative T_2 contrast agents, the presence of inherent hypointense regions and possible haemorrhages make MRI results difficult to interpret. Nevertheless, the development of positive contrast using SPIO (48,49) or MRI reporter genes such as ferritin (50) could help to resolve these issues. Finally, the high sensitivity of BLI and the high resolution of conventional MRI both allow long-term monitoring of metastatic cells and accurate localization of macrometastases.

5. Conflicts of Interest and Source of Funding

This work was supported by grants from the Fonds National de la Recherche Scientifique (FRS-FNRS), the Fonds Joseph Maisin, the

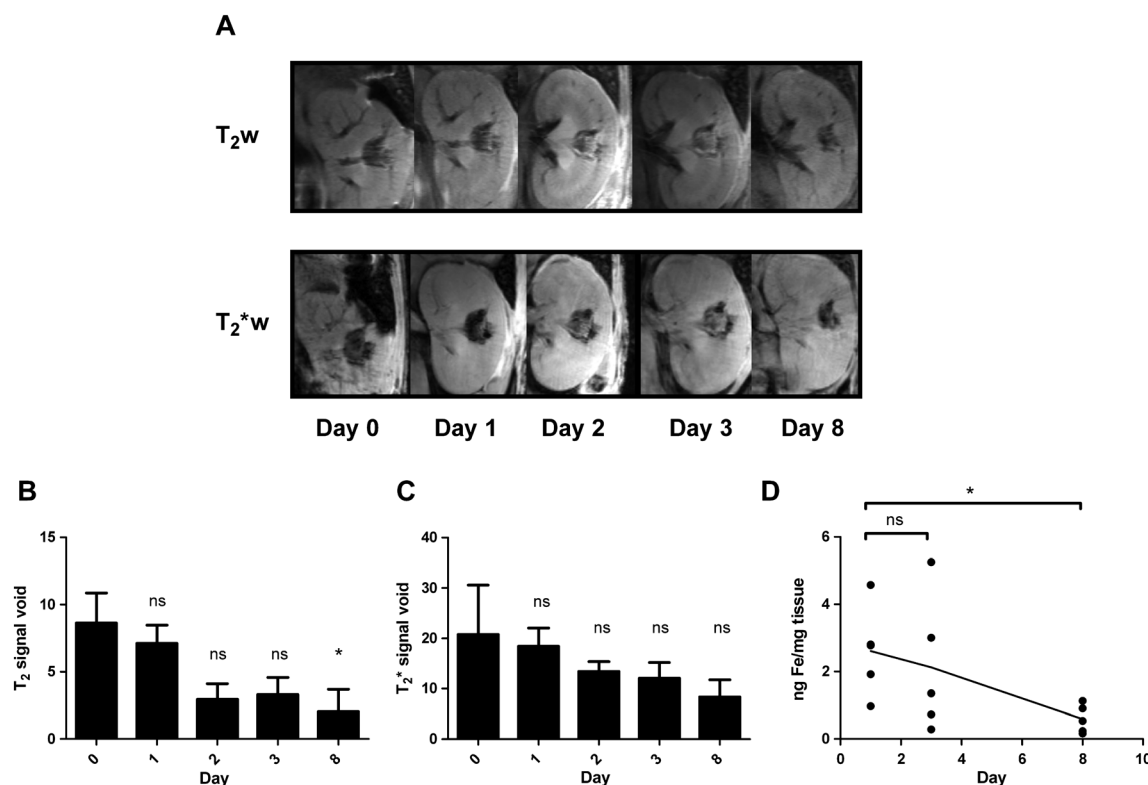


Figure 9. MR monitoring of free MIRB iron oxide nanoparticles after injection in the left kidney. (A) Representative coronal images (T_2w and T_2^*w) of the left kidney on days 0, 1, 2, 3 and 8 after injection of free MIRB ($3 \mu\text{g Fe}$). Signal voids on T_2w (B) and T_2^*w (C) tended to decrease with time, but hypointense regions persisted up to 8 days after injection ($n=4$). (D) The intrarenal iron oxide content after injection of $1 \mu\text{g Fe}$ MIRB decreased with time, but SPIO were still detected 8 days after injection. Data are represented as means $\pm$ SEM except for (D), where each individual measurement is represented; medians are crossed by the connecting curve. ns, $p > 0.05$, * $p < 0.05$.

Saint-Luc Foundation, the Fondation contre le Cancer, the 'Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020', and the European Research Council (FP7/2007-2013 ERC Independent Researcher Starting Grant no. 243188 TUMETABO to P. Sonveaux). B.F. Jordan and P. Sonveaux are Research Associates and P. Danhier is a Research Fellow of the FRS-FNRS. G. De Preter is a Télévie Research Fellow. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors have declared that no competing interests exist.

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III. *In vivo* visualization and *ex vivo* quantification of murine cancer cells in the brain using MRI cell tracking and electron paramagnetic resonance

Pierre Danhier, Julie Magat, Géraldine De Preter, Paolo E. Porporato, Caroline Bouzin, Bénédicte F. Jordan, Olivier Feron, Pierre Sonveaux and Bernard Gallez

Ex vivo EPR, performed for measuring the iron oxide content of freeze-dried organs, appeared in the previous study to be the most reliable method for assessing the short-term fate of tumor cells after delivery, whereas MRI and BLI were more adapted for assessing late metastases. However, several questions remained: is it possible to accurately determine how much cells arrive at a distant site? Is it possible to visualize (U)SPIO-labeled cells using MRI in other organs than the liver? As single imaging of SPIO-labeled cells was reported in the mouse brain, we investigated the complementarity of EPR and MRI for assessing the homing of murine breast cancer cells to the brain.

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***In Vivo* Visualization and *Ex Vivo* Quantification of Murine Cancer Cells in the Brain Using MRI Cell Tracking and Electron Paramagnetic Resonance**

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Running title: Cell tracking of brain metastases using EPR and MRI

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Abstract

Purpose: The aim of this study was to visualize, using MRI (11.7 T), and quantify, using electron paramagnetic resonance (EPR), the entrapment of murine breast cancer cells labeled with superparamagnetic iron oxide particles (SPIO) in the brain after intracardiac injection.

Methods: Murine 4T1 breast cancer cells were labeled with fluorescent Molday Ion Rhodamine B SPIO. Following intracardiac injection, SPIO-labeled 4T1 cells were imaged using multi gradient-echo sequences. *Ex vivo* iron oxide quantification in the mouse brain was performed using X-band EPR (9 GHz).

Results: Hypointense spots due to SPIO-labeled cells were visualized 4 h after injection. Quantification of SPIO using EPR showed that <1% of the injected cells were taken up by the mouse brain after injection. Immunofluorescence studies demonstrated that these cells found in the brain established micrometastases several days after injection.

Conclusion: EPR and MRI were found to be complementary for cell-tracking applications. MRI cell tracking at 11.7 T allowed sensitive detection of isolated SPIO-labeled cells, whereas EPR allowed an accurate assessment of the number of SPIO-labeled cells in the mouse brain shortly after injection. The absolute quantification of SPIO using EPR could be useful for other cell-tracking applications such as cell-based therapies.

Keywords: electron paramagnetic resonance, magnetic resonance imaging, cell tracking, cancer, metastasis, iron oxide particles

Introduction

It is necessary to monitor the fate of cells of interest after administration for the successful application of cell-based therapies and the understanding of fundamental processes in cancer biology (e.g., metastasis) (1). *In vivo* MRI cell tracking has been extensively developed for such applications and consists of visualizing cells previously labeled with contrast agents. Superparamagnetic iron oxide particles (SPIO) are widely used because they induce signal voids on T_2 -, T_2^* -weighted images and are biocompatible (2). Although developments have led to single-cell detection (3,4), quantitative methods are nevertheless needed to accurately assess the distribution of labeled cells in living organisms. Non-invasive quantification methods mostly rely on relaxometry-based techniques or retrieval of information on magnetic field inhomogeneities from phase maps (5). Relaxometry-based techniques are not directly applicable *in vivo* because physiological processes such as SPIO compartmentalization, cell division, and particle release from the cell affect the relaxation properties of SPIO (6,7). Furthermore, Rad *et al.* have shown that different cell lines labeled with the same particles and having the same intracellular iron oxide content exhibit different relaxation rates (8). A recent method based on modeling the magnetic field inhomogeneities induced by SPIO showed accurate estimates of iron oxide content in phantoms and *in vivo*. However, this model was sensitive to tissue-tissue and air-tissue interfaces and assumed that the SPIO distribution was homogenous in the region of interest (5).

In the present study using high field MRI (11.7 T), we aimed to track 4T1 breast cancer cells labeled with Molday Ion Rhodamine B (MIRB) SPIO (diameter = 35 nm), initially injected into the heart, in the mouse brain. 4T1 cells have been described as establishing brain metastases after intracardiac injection (9,10). MIRB iron oxide nanoparticles (colloidal size of 35nm) were shown to be nontoxic and to be retained within the cell cytoplasm after labeling (11,12). The iron oxide content in the brains was assessed *ex vivo* using electron paramagnetic resonance (EPR). Although animal sacrifice is required, this technique was used in several studies for absolute SPIO quantification after injection (13-16). Moreover, *ex vivo* EPR is specific for iron oxides and is not influenced by the presence of endogenous iron (17). Using both MRI for SPIO-labeled cells visualization and EPR for SPIO quantification, we wanted to assess the complementarity of both methods for cell-tracking studies.

Methods

Cell culture

Luciferase-expressing 4T1-luc murine breast cancer cells were grown in DMEM + GlutaMAX (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen).

Cell labeling with SPIO

Two days prior to the experiment, 4T1-luc cells were seeded at a concentration of 10^4 cells/cm². The next day, cells were incubated overnight with MIRB particles (Biopal, Worcester, USA) in full medium at a concentration of 20 µg Fe/mL. After labeling, cells were washed three times with PBS, trypsinized, centrifuged and resuspended in PBS. Cells were then counted using a hemocytometer. Viable cells (assessed using Trypan Blue dye) were centrifuged again and kept on ice in a PBS solution.

Intracellular iron oxide content quantification and SPIO localization after labelling

The intracellular iron oxide content per cell was determined using EPR as previously described (11). Fluorescence microscopy was performed to assess the cellular localization of red fluorescent MIRB nanoparticles after labeling. Briefly, cells were seeded on Lab-Tek Chamber Slides (Sigma) and incubated overnight in full medium with MIRB particles (20 µg Fe/mL). Cells were rinsed three times with PBS, fixed with paraformaldehyde 4% (Sigma) and permeabilized with PBS/0.1% Triton. Actin was stained using phalloidin-FITC (Sigma). Slides were then mounted using Vectashield H1000 (Vector Labs, Brussels, Belgium) and analyzed using a Zeiss Axio Imager fluorescence microscope equipped with an ApoTome module (structured illumination).

Ethical statement

All *in vivo* experiments were performed in compliance with guidelines set by national regulations and were approved by the ethical committee for animal care of the Université catholique de Louvain.

Intracardiac injection of 4T1-luc cells

Five-week-old female Balb/c mice (Janvier) were anesthetized with ketamine (80 mg/kg)/xylazine (8 mg/kg). A small skin incision was made to expose the third intercostal space; 100 μ L of PBS containing MIRB-labeled 4T1-luc cells or free MIRB particles were then slowly injected into the left ventricle using an insulin syringe. The superficial incision was then sutured.

Magnetic Resonance Imaging

In vivo MR experiments on mouse brains were performed with an 11.7 T system (Bruker, Biospec) equipped with a cryoprobe. Animals were anesthetized by inhalation of 1.5 to 2% isoflurane (Forene, Abbot, England). Mouse temperature was maintained at 37.0 °C using a water blanket connected to a circulating water bath, and respiration was monitored. Coronal T₂-weighted (T₂-w) images were acquired using a Turbo RARE sequence with the following parameters: TR, 2500 ms; TE, 39.1 ms; flip angle, 180°; RARE factor, 8; field of view, 2x2 cm; matrix, 350x350; slice thickness, 1 mm; in-plane resolution, 57x57 μ m. Coronal T₂*-weighted (T₂*-w) images were acquired using a Multi Gradient-Echo (MGE) sequence with the following parameters: TR, 2000 ms; TE, 5 ms; excitation pulse angle, 30°; field of view, 2x2 cm; matrix, 350x350; slice thickness, 1 mm; in-plane resolution, 57x57 μ m. *In vitro* MRI was performed on 4% gelatin phantoms containing 3x10⁵ cells/mL using MGE sequences with the same parameters used for *in vivo* imaging (18).

Histology

Four hours after intracardiac injection of MIRB-labeled 4T1-luc cells (2x10⁶ cells), *in vivo* MRI was performed to detect labeled cells in the brain. MRI positive mice were sacrificed and the brains were collected. After overnight fixation at 4°C in 4% paraformaldehyde, the brains were embedded in paraffin and 5 μ m coronal sections were made. Iron oxide presence was revealed using Prussian blue staining and nuclear fast red counterstaining.

To assess the presence of 4T1-luc micrometastases, mice were injected in the left ventricle with 10⁶ MIRB-labeled cells and underwent MRI scans. MRI positive mice were sacrificed 13 days post-injection and the brains were embedded in OCT compound (Gentaur)

and frozen with liquid nitrogen vapor; 10 μ m coronal sections were made using a cryostat. Sections were fixed for 10 minutes in cold acetone. After a 1 hour blocking step with PBS/0.1% Tween 20 (PBST) containing 5% bovine serum albumin (BSA), sections were incubated with a rabbit anti-luciferase antibody (1/1000 overnight at 4°C in PBST/BSA 1%, Abcam). Luciferase-expressing cells were revealed with an Alexa 488 mouse anti-rabbit secondary antibody (1 hour, 1/500 at 37°C in PBST/BSA 1%, Invitrogen). Nuclei were stained using Hoechst 33342 (Sigma).

***Ex vivo* iron oxide content determination using X-band EPR**

Ex vivo iron oxide content in the brains, livers and spleens of mice injected in the left ventricle with 10⁶ MIRB-labeled 4T1-luc cells, or free MIRB nanoparticles (5 μ g Fe) as a control, was determined using X-band EPR (9 GHz). After animal sacrifice, the organs were harvested, freeze-dried and crushed into a fine powder. Dry samples were then weighed and analyzed using EPR with the following settings: microwave power, 5.05 mW; center field, 3150 G; field sweep width, 5000 G; modulation amplitude, 30 G; time constant, 20.48 ms; conversion time, 20.48 ms; modulation field, 100 kHz. Measurements were performed at room temperature. The iron oxide content in each freeze-dried organ was quantified by comparison with a calibration curve of dry MIRB standards. The limit of detection (LOD) and quantification (LOQ) of *ex vivo* EPR were assessed using a calibration curve for different concentrations of MIRB-labeled cells after water removal (oven at 60°C for 3 days).

Statistical analyses

All results are represented as means $\pm$ standard error of the mean (SEM). Comparisons between groups were analyzed using Student's *t*-tests; $p < 0.05$ was considered to be statistically significant.

Results

Sensitivity of *ex vivo* EPR for iron oxide content determination

We first attempted to determine the sensitivity of EPR in quantifying SPIO-labeled cells in organs. Fluorescence microscopy images (Figure 1A) showed that 4T1 cells efficiently internalized fluorescent MIRB particles. SPIO were localized within the cytoplasm after incubation. After labeling, 4.62 ± 0.26 pg Fe were internalized per cell (n=3).

We next assessed the sensitivity of EPR in detecting iron oxides *ex vivo*. Calibration curves recorded with increasing concentrations of water-free MIRB-labeled cells showed that EPR could detect 2100 MIRB-labeled cells and that the LOQ was equal to 7200 MIRB-labeled cells (Figure 1B). The LOD and LOQ of *ex vivo* EPR were calculated using the formulas $LOD=3SD/a$ and $LOQ=10SD/a$ (SD= standard deviation, a= slope of the calibration curve).

In vivo MR imaging of isolated SPIO-labeled cells in the brain

In vivo MRI was then performed to monitor the fate of MIRB-labeled breast cancer cells in the mouse brain after intracardiac injection. As shown in Figure 2A, numerous hypointense spots were observed on T_2^* -w images 4 hours after injection of 10^6 MIRB-labeled 4T1 cells), whereas anatomical T_2 -w images did not display these features. These hypointense spots decreased with time and became undetectable 3 days post-injection (Figure 2B). In a control experiment, T_2^* -w images of gelatin phantoms containing MIRB-labeled cells (3×10^5 cells/mL) displayed numerous signal voids, whereas unlabeled 4T1 cells at the same concentration did not induce negative contrast on MR images (Figure 2C). This concentration of cells was chosen to ensure a cell density of 1 cell/voxel. Prussian blue staining performed after brain imaging on day 0 confirmed the presence of iron-positive cells (Figure 3). Interestingly, only isolated Prussian blue positive cells or small clusters of cells were found within the brain tissue.

Iron oxide quantification in the brain after intracardiac injection of SPIO-labeled cells

Having visualized isolated MIRB-labeled 4T1 cells using MRI at 11.7 T, we next wanted to know how many SPIO-labeled cancer cells were entrapped in the mouse brain after

intracardiac injection. Figure 4A shows that hypointense spots could be detected on T_2^* -w images 4 hours after injection of 10^6 MIRB-labeled cells, whereas no negative contrast was observed when mice received free MIRB particles (corresponding to 5 μ g Fe). This dose corresponded to the iron content of 10^6 MIRB-labeled cells. After MR imaging, the iron oxide content of brains, livers and spleens was assessed using *ex vivo* EPR. Significant levels of iron oxides were found in the brains of mice injected with SPIO-labeled 4T1 cells, whereas a negligible EPR signal was detected in the brains of control mice (Figure 4B, 0.52 ± 0.11 ng Fe/mg brain tissue for the 4T1 + MIRB group *versus* 0.01 ± 0.00 ng Fe/mg tissue for the MIRB group). We next assessed the iron oxide content in organs involved in iron oxide metabolism, i.e., the spleen and the liver. Four hours after intracardiac injection, iron oxide levels in the liver were 3.83 ± 0.57 ng Fe/mg tissue for mice injected with SPIO-labeled cells and 8.65 ± 1.21 ng Fe/mg tissue for control mice that received free MIRB (Figure 4C). Similarly, higher iron oxide levels were found in the spleens of mice injected with free SPIO in comparison with the 4T1 + MIRB group (Figure 4D, 8.59 ± 1.55 ng Fe/mg tissue for the 4T1 + MIRB group *versus* 43.06 ± 1.46 ng Fe/mg tissue for the MIRB group). One day post-injection, the iron oxide content dropped dramatically in the brain and a statistical difference between groups was no longer observed (0.14 ± 0.07 ng Fe/mg tissue for the 4T1 + MIRB group *versus* 0.02 ± 0.01 ng Fe/mg tissue for the MIRB group, $n=4$ for each group).

Long-term assessment of the fate of SPIO-labeled murine breast cancer cells in the brain

After visualizing *in vivo* and quantifying *ex vivo* the number of SPIO-labeled 4T1 cells localized in the brain 4 hours after injection, we addressed the question of whether a subset of these entrapped cancer cells could lead to the formation of metastases (Figure 5). Figure 5A shows that no macrometastases could be observed on conventional T_2 -w images 10 days after intracardiac injection of 0.75×10^6 4T1 cells. On the other hand, immunofluorescence images (Figure 5B) highlighted the presence of luciferase-positive micrometastases 13 days after injection of 10^6 MIRB-labeled 4T1-luc cells. Micrometastases were found in the brain parenchyma in the same regions where signal voids were detected on day 0 on T_2^* -w images (Figure 2).

Discussion

Using MRI and *ex vivo* EPR, we succeeded in visualizing and quantifying SPIO-labeled circulating tumor cells entrapped in the mouse brain after intracardiac injection. As demonstrated with phantom studies, isolated cells and small clusters of SPIO-labeled cells were detected. Nevertheless, MR images showed that signal voids due to SPIO-labeled cells decreased in the following days post-injection. This can be explained by the dilution of the intracellular SPIO content with cell proliferation (19), SPIO metabolism (20,21) and clearance of SPIO released by dead cells from the brain (22). It was also shown using EPR that the iron oxide content in the mouse brains dropped below the detection threshold one day post-injection. This decrease is probably due only to the clearance and degradation of SPIO. The dilution of iron oxide particles due to cell proliferation should not affect the total iron oxide content in the brain. Despite a loss of contrast on MR images and a drop in SPIO content, a subset of MIRB-labeled cancer cells survived and led to the formation of micrometastases.

Although other studies have reported the use of MRI cell tracking to study brain metastasis formation after intracardiac injection of cancer cells (22-24), in this work we report for the first time accurate quantification of SPIO in the mouse brain after administration of murine breast cancer cells labeled with SPIO. Using EPR, we can estimate that ~8000 (<1%) of the injected cells (10^6) were found in the brain 4 hours after intracardiac injection (dry weight of the brain ~80mg, injection of 10^6 MIRB-labeled 4T1 cells containing 5 pg Fe, ~500 pg Fe/mg brain tissue 4 hours after injection). Considering that blood flow to the mouse brain represents only 3% of the cardiac output (25), our results indicate that one quarter of the circulating tumor cells in the brain were entrapped 4 hours after injection. These calculations assume that cells did not start to proliferate after injection (4 h).

MRI was shown to precisely localize labeled cancer cells in the brain, but the number of hypointense spots did not necessarily correspond to the number of cells. As shown with Prussian blue staining histology, signal voids on gradient-echo images could be due to isolated SPIO-labeled cells, but also small clusters of SPIO-labeled cells. Visualization of SPIO-labeled cells on T_2^* -w images is also hampered by artifacts due to air-tissue interfaces. Non-invasive quantification of SPIO for cell tracking applications remains challenging because the presence of endogenous iron, SPIO particles released from labeled cells, clustering and distribution of the SPIO within the tissue are factors that strongly influence the relaxation properties of iron oxides (5,26). To our knowledge, successful *in vivo*

quantification has been reported in one study after intra-articular administration of bone marrow-derived mesenchymal stem cells labeled with SPIO. In this study, cells were localized in a confined space and their exact number was known (27). On the other hand, EPR has been shown in several studies to be a robust method for assessing the fate of SPIO after administration in living animals (17,28). As a consequence, the quantitative information provided by the EPR method might be useful in development of new *in vivo* methods for SPIO quantification.

In conclusion, *ex vivo* EPR and MRI are complementary methods for cell tracking applications. In our study, a correct estimate of the number of labeled cells using EPR was only possible a few hours after injection. Owing to the highly proliferative phenotype of cancer cells, the iron oxide content per cell would have been modified in the next days following administration. Nevertheless, cell-based therapies using cells with lower proliferative potential constitute a main field of applications for MRI cell tracking (29). For such studies, EPR could be complementary to MRI in assessing the biodistribution of SPIO-labeled cells for prolonged periods after administration.

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Figures

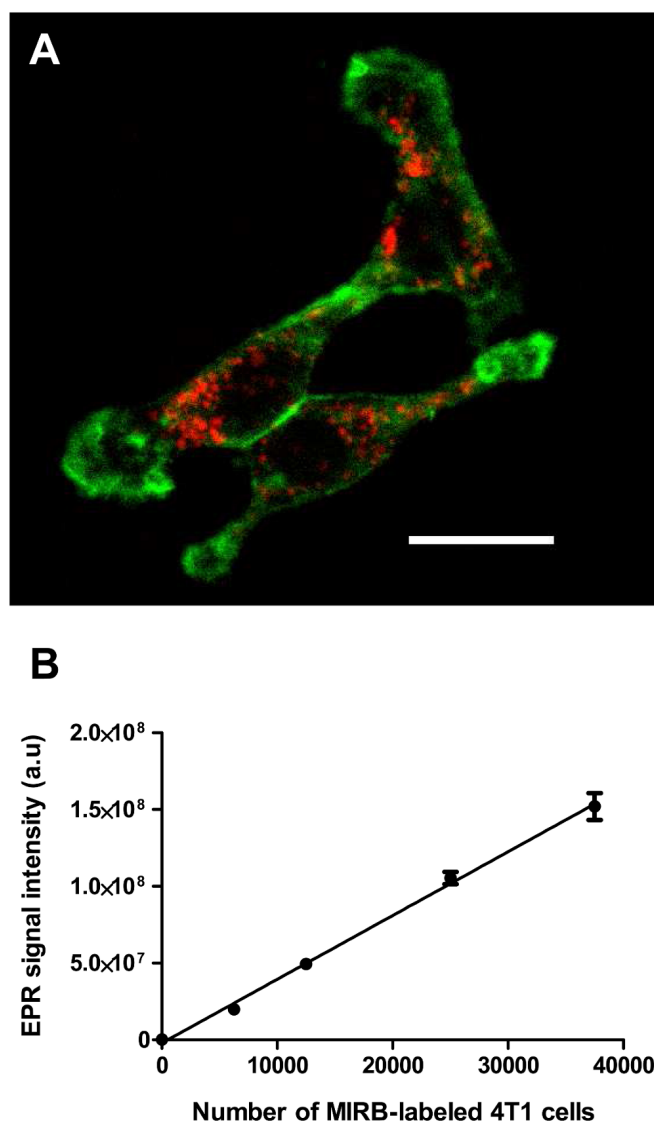


Figure 1. EPR detection of 4T1 cells labeled with MIRB nanoparticles. (A) Fluorescence microscopy of 4T1 cells labeled with Molday Ion Rhodamine B (red fluorescence). Actin is stained in green using phalloidin-FITC. The scale bar represents 20 μm (B) Linear regression of increasing amounts of MIRB-labeled cells after water removal using X-band EPR (n=4). Data are expressed as means ± SEM.

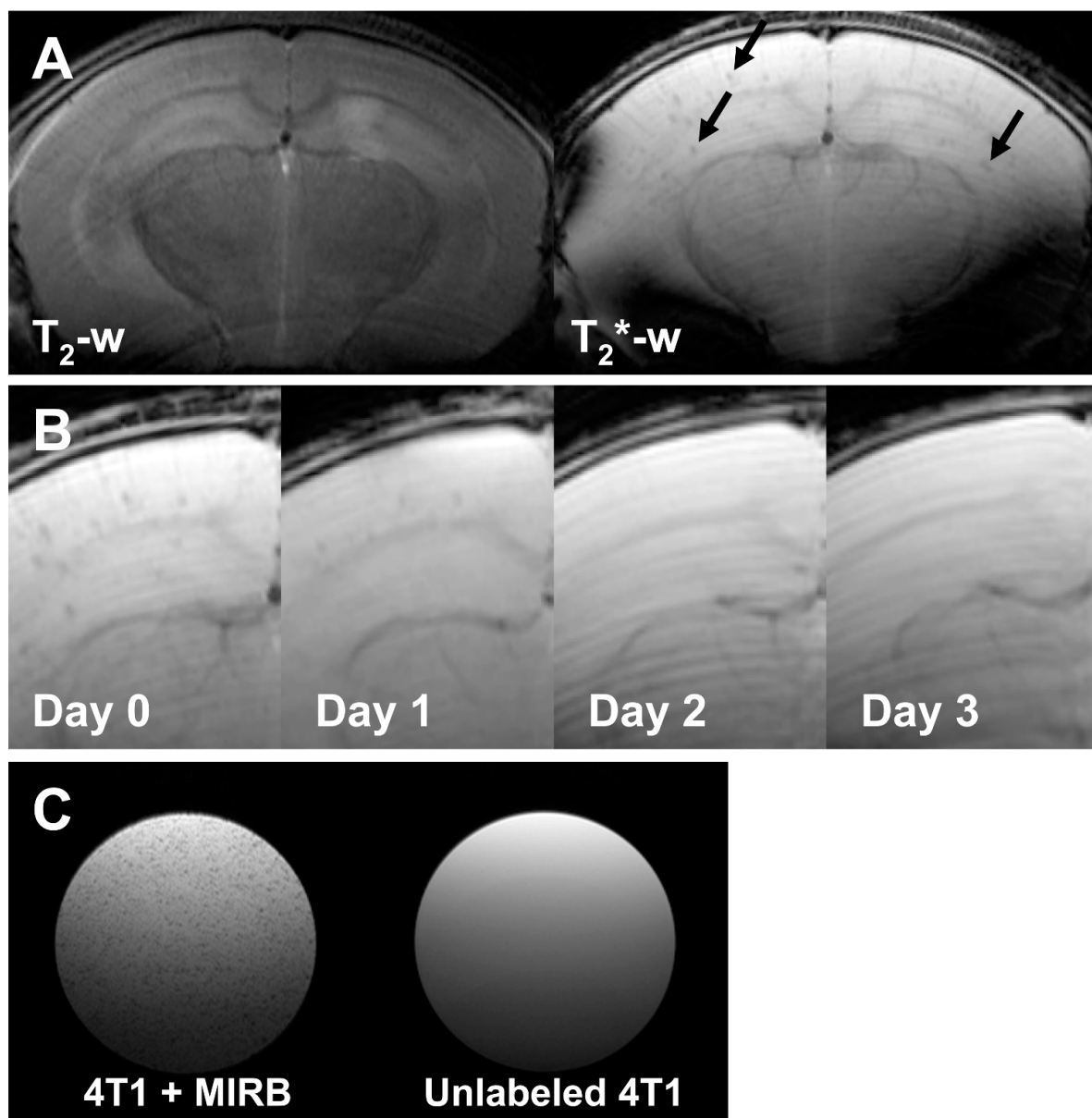


Figure 2. MR visualization of MIRB-labeled 4T1 cells 4 hours after intracardiac injection. (A) Signal voids were detected on T₂*-w images 4 hours after injection of 10⁶ MIRB-labeled 4T1 cells, whereas no hypointensities were observed on T₂-w images (n=3). Typical signal voids are indicated by black arrows (B). A progressive loss of signal voids was observed in the days after injection. (C) T₂*-w images of gelatin (4%) phantoms containing MIRB-labeled 4T1 cells or unlabeled cells showed that signal voids were due to MIRB-labeled cells (n=3 for each group).

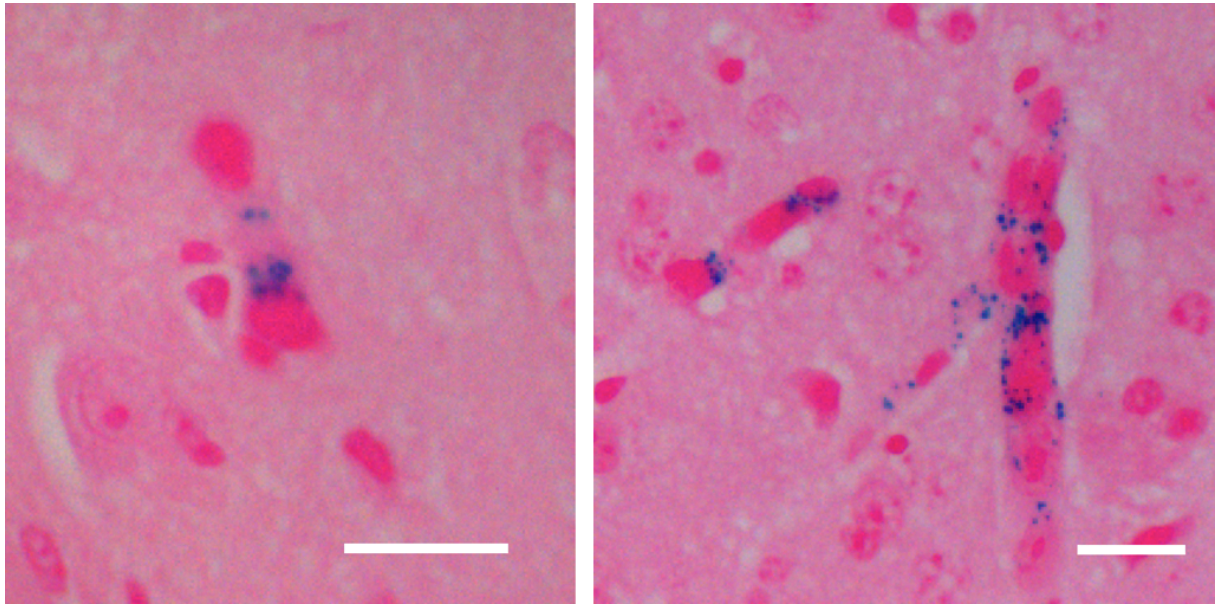


Figure 3. Histological confirmation of the presence of iron oxides in mouse brains after MRI. Prussian blue staining confirmed the presence of isolated (left picture) or small clusters of iron-positive cells (right picture) 4 hours after intracardiac injection of MIRB-labeled 4T1 cells. The scale bar represents 20 μm .

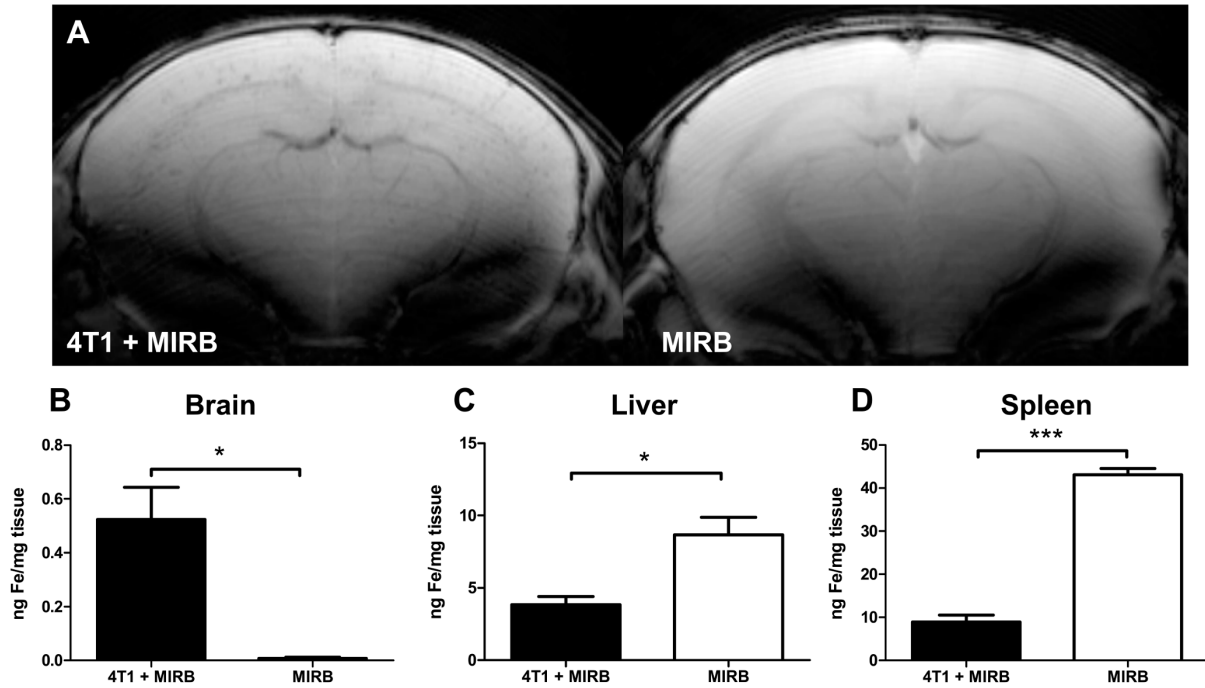


Figure 4. Correlation between MR detection of MIRB-labeled 4T1 cells and *ex vivo* EPR quantification of iron oxides in mouse brains. (A) MR images produced 4 hours after administration of 10^6 MIRB-labeled cells or 5 μ g Fe of free MIRB particles. No signal voids were detected in mice receiving free MIRB (n=3 for each group). (B) After MRI, a major difference in iron oxide content in the brain was found between mice injected with MIRB-labeled cells and mice injected with free MIRB. On the other hand, higher iron oxide amounts were found in the liver (C) and the spleen (D) of mice injected with free MIRB particles (n=3 for each group). Results are statistically significant. Data are expressed as means $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$

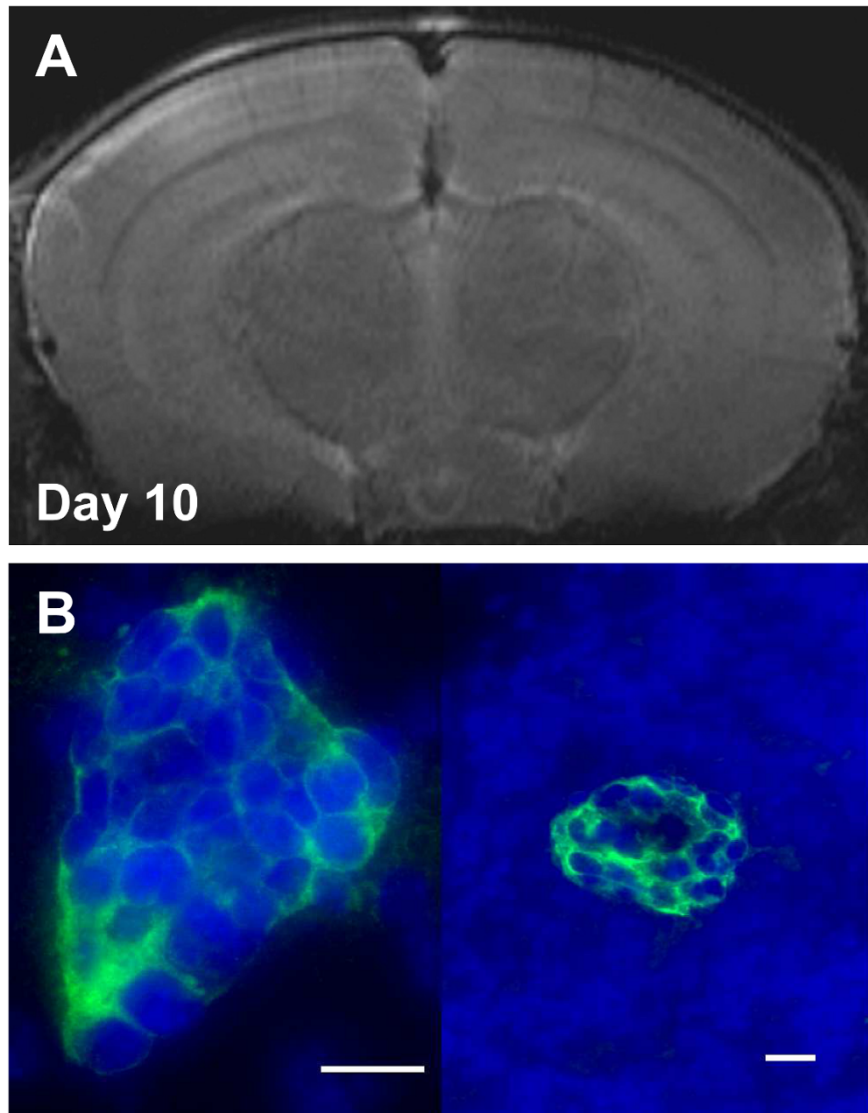


Figure 5. Long-term assessment of the presence of 4T1-luc cells in mouse brains following intracardiac administration. (A) No macrometastases could be detected using anatomical T₂-w images (n=3), whereas immunofluorescence studies (B) demonstrated the presence of 4T1-luc micrometastases 13 days after injection of 4T1-luc cells (n=3). Luciferase-positive cells are stained in green and nuclei are stained in blue using Hoechst 33342. The scale bar represents 20 μm.

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IV. Influence of cell detachment on the respiration rate of tumor and endothelial cells

Pierre Danhier, Tamara Copetti, Géraldine De Preter, Philippe Leveque, Olivier Feron, Bénédicte F. Jordan, Pierre Sonveaux and Bernard Gallez

Despite they provide important information on cell localization, SPIO-based cell tracking methods do not give any information on cell function. In the context of invasion metastasis, the metabolism of circulating tumor cells is poorly understood. Therefore, metabolic changes occurring after cell detachment were assessed.

Influence of Cell Detachment on the Respiration Rate of Tumor and Endothelial Cells

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Abstract

Cell detachment is a procedure routinely performed in cell culture and a necessary step in many biochemical assays including the determination of oxygen consumption rates (OCR) *in vitro*. *In vivo*, cell detachment has been shown to exert profound metabolic influences notably in cancer but also in other pathologies, such as retinal detachment for example. In the present study, we developed and validated a new technique combining electron paramagnetic resonance (EPR) oximetry and the use of cytodex 1 and collagen-coated cytodex 3 dextran microbeads, which allowed the unprecedented comparison of the OCR of adherent and detached cells with high sensitivity. Hence, we demonstrated that both B16F10 melanoma cells and human umbilical vein endothelial cells (HUVEC) experience strong OCR decrease upon trypsin or collagenase treatments. The reduction of cell oxygen consumption was more pronounced with a trypsin compared to a collagenase treatment. Cells remaining in suspension also encounter a marked intracellular ATP depletion and an increase in the lactate production/glucose uptake ratio. These findings highlight the important influence exerted by cell adhesion/detachment on cell respiration, which can be probed with the unprecedented experimental assay that was developed and validated in this study.

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Introduction

The oxygen consumption rate (OCR) of cells is an important biological parameter studied in various fields as an indicator of mitochondrial activity. In tumor biology, cancer metabolism studies and OCR measurements have been decisive to show that a decrease in mitochondrial respiration and enhanced aerobic glycolysis (known as the Warburg effect) are linked with tumor aggressiveness [1–3], and that endothelial cells submitted to hypoxia undergo profound metabolic changes [4]. OCR is also widely assessed in tumor treatment response and was instrumental to demonstrate that a blockade of mitochondrial respiration, supporting an increase in the local pO₂, is a valid combinatory treatment option to improve chemo- and radiosensitivity [5–9]. Concerning other pathologies, oxygen metabolism is investigated in retinal detachment where restoring appropriate oxygenation can prevent vision loss [10,11]. More generally, OCR is also an accepted viability marker used for tissue transplantation [12].

Several techniques are currently used for measuring OCR *in vitro*. The most common ones rely on the use of polarographic Clark electrodes, phosphorescent or fluorescent probes, or electron paramagnetic resonance (EPR) [13,14], whereas other methods (based on spectrophotometry or near-infrared spectroscopy for example) have been described but are less used [15,16]. It has

been recently demonstrated that EPR is much more sensitive than fluorescence assays or the use of Clark electrodes [13], which is directly attributable to the physics of EPR oximetry as extensively described in recent comprehensive reviews [17,18]. Briefly, OCR determination using EPR oximetry is based on the signal variation of a paramagnetic oxygen sensing probe in contact with cells sealed in a glass capillary tube (Figure 1). In this setting, cells consume O₂ at a rate mainly dictated by their mitochondrial activity.

The present study is based on previous works having demonstrated *in vivo* that cell detachment promotes tumorigenesis and leads to metabolic alterations reflected by decreased glucose uptake and decreased ATP levels [19]. A similar metabolic reprogramming could therefore impose a signal drift on the most commonly used OCR assays that generally involve cell harvesting for technical reasons [20–22]. Hence, characterizing the impact of cell adhesion and detachment on OCR might also lead to a better understanding of cell-cell and cell-surface interactions, which necessarily involves the design and validation of an appropriate OCR measurement technology. To reach that goal, we aimed to set up and validate a new protocol combining the high sensitivity of EPR and the use of cytodex microcarriers in order to study the influence of cell adhesion and detachment on OCR *in vitro*. Cytodex 1 and collagen-coated cytodex 3 carriers consist in 100-

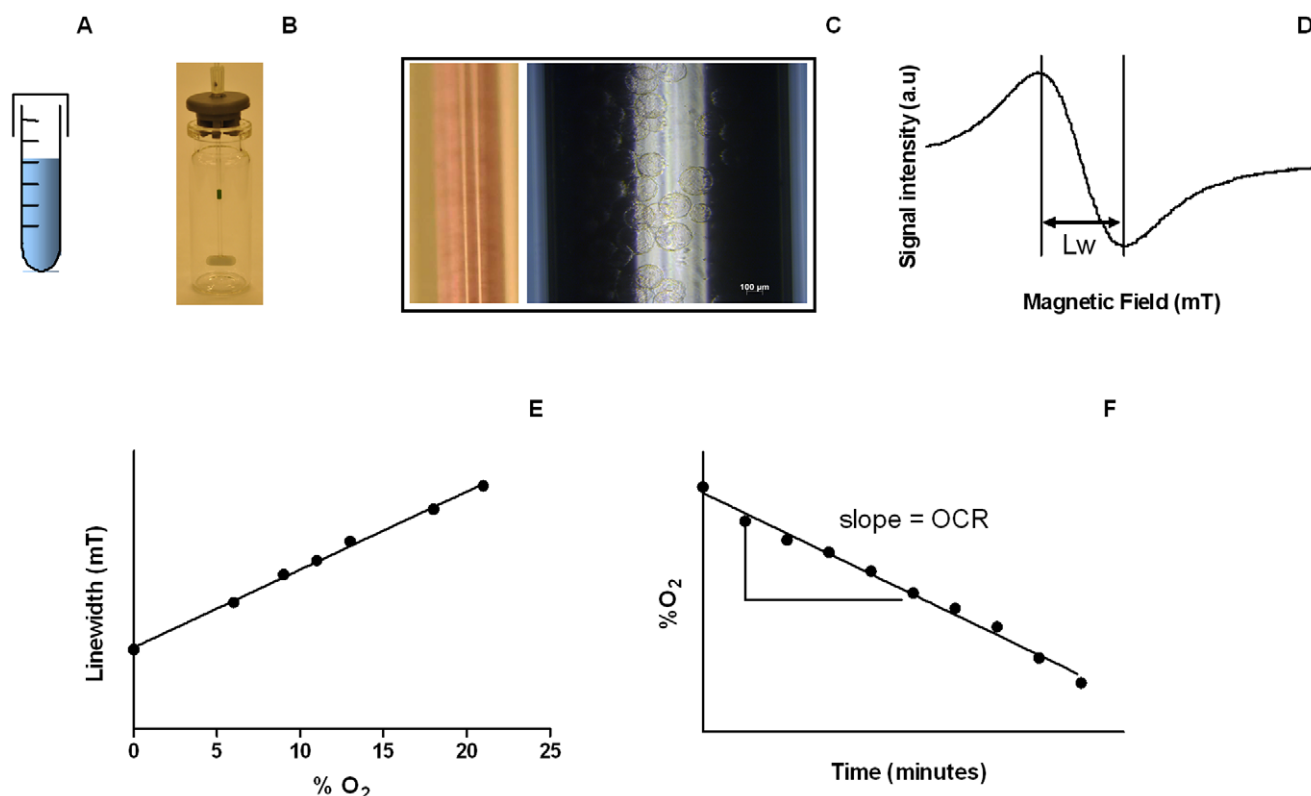


Figure 1. Experimental setup. (A) Cells were incubated with cytodex microbeads during 4 hours at 37°C. (B) Cell-coated microcarriers were placed in a thermostated (37°C) minispinner in a medium containing 10% (w/v) dextran. (C) Cell-covered microbeads were mixed with a nitroxide (^{15}N -PDT) oxygen probe and drawn into a capillary tube which was then sealed, this last one was inserted in the EPR X-band cavity for measurements (D) EPR spectra of ^{15}N -PDT were recorded, the linewidth (Lw) being proportional to %O₂. (E). (F) Recorded linewidths allowed determining the evolution of %O₂ inside the closed capillary containing adherent cells. The slope is actually the oxygen consumption rate (OCR).
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μm diameter dextran microbeads on which cells can easily adhere and grow [23]. Adherent cells can then be handled similarly to cells in suspension, implying that, once carefully validated with microbeads, EPR oximetry could be amenable for the OCR measurement of intact adherent cells.

Materials and Methods

Layout of the General Setup

The general objective of the experimental approach is to measure the OCR of two intact, adherent cell lines, namely tumor and endothelial cells, which represent two major cell populations in tumors. The impact of cell detachment on OCR will be studied using two modalities used in classical metabolomics, i.e. the use of trypsin or collagenase. The general strategy is displayed in Figure 1. Cells were coated onto cytodex microcarriers. On the next day, cells on microbeads were poured into a homemade minispinner (adapted from [24]) in a dextran-rich medium at 37°C to avoid sedimentation. Cells were then carefully aspirated into an eppendorf cup (treated with trypsin or collagenase for the detached group), mixed with an EPR oxygen-sensing nitroxide probe, and drawn into a glass capillary tube for EPR measurements. Trypsin or collagenase treatment modalities are summarized in Table 1.

Cell Culture

Murine melanoma B16F10-luc cells constitutively expressing luciferase (Xenogen) were grown in Dulbecco's Modified Eagle

Medium (containing GlutaMAXTM I, 1000 mg/L *D*-glucose, sodium pyruvate), supplemented with 10% fetal bovine serum, and 1% (v/v) penicillin/streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVECs, Sigma-Aldrich) were grown in the Endothelial Cell Growth Medium of Sigma-Aldrich.

Cell Loading onto Cytodex Microbeads

On the day prior to the experiment, confluent cells were rinsed with PBS, harvested using 0.05% Trypsin-EDTA (Invitrogen), and admixed with full medium (1:1). After centrifugation (180 g, 5 min), the cells were resuspended in full medium containing sterilized Cytodex 1 (30 mg for B16F10-luc) or collagen-coated Cytodex 3 dextran (30 mg for B16F10-luc, 65 mg for HUVEC) microbeads (Sigma-Aldrich) in a 17*100 mm round bottom tube (BD Biosciences). The tube was then placed during 4 hours in an incubator at 37°C and flicked every twenty minutes during incubation to allow uniform anchorage and distribution of the cells onto the microbeads. After incubation, cell-coated microbeads were placed overnight in a T75 flask (Sarstedt) with full medium at 37°C/5%CO₂.

Adherent Cell Preparation for EPR Oximetry

Cells on dextran microbeads were collected and placed into a round bottom tube during 3 minutes in order to settle. After medium removal, the cell-coated beads were washed gently with 10 ml of PBS. A 100 μl aliquot of the suspension was collected, diluted 10 times with PBS in a microtube, and three 10 μl aliquots of this suspension were used to determine the bead concentration

Table 1. Summary of the different experimental conditions.

Cell line	Group	Detachment procedure
B16F10-luc	Control	Microbeads covered by cells in full medium with dextran 10% (w/v) (final volume: 300 μ l)
B16F10-luc	Trypsin	0.05% Trypsin-EDTA 10 minutes at 37°C followed by addition of full medium with dextran 20% (w/v) (final volume: 300 μ l)
B16F10-luc	Collagenase	Collagenase (10 mg/ml) 15 minutes at 37°C (final volume: 300 μ l)
HUVEC	Control	Microbeads covered by cells in full medium with dextran 10% (final volume: 100 μ l)
HUVEC	Trypsin	0.05% Trypsin-EDTA diluted 2.5 times 5 minutes at 37°C followed by addition of full medium with dextran 20% (w/v) (final volume: 100 μ l)
HUVEC	Collagenase	Collagenase (10 mg/ml) 15 minutes at 37°C with dextran 20% (w/v) (final volume: 100 μ l)

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under an inverted microscope. Another 100 μ l sample was then collected from the 10 ml suspension, mixed with 360 μ l 0.05% Trypsin-EDTA and a 40 μ l dextranase solution (Sigma-Aldrich) for dissolving the beads (15 minutes at 37°C with continuous agitation) [25]. Released cells were then counted using a hemocytometer. The PBS supernatant of the stock suspension was discarded and replaced by 2 ml of full medium containing 10% (w/v) dextran (molecular weight: 64000–76000) (Sigma-Aldrich). This final suspension was carefully transferred into a thermostated (37°C) homemade minispinner flask with 40 rpm agitation as already reported [24]. This procedure allows the cells to remain intact and adherent for several hours. Each experiment was performed with cells coming from the same minispinner, implying that cell concentration was exactly the same for control (adherent) and treated (trypsin or collagenase) groups. For oxygen measurements with adherent B16F10-luc cells, 100 μ l of cell-coated microbeads were mixed with 190 μ l of full medium containing 10% (w/v) dextran and 10 μ l of 2 mM oxygen-sensing probe ^{15}N -4-oxo-2,2,6,6-tetramethylpiperidine- d_{16} - ^{15}N -1-oxyl (^{15}N -PDT). For adherent HUVECs, 95 μ l of the microbead solution were mixed with 5 μ l of ^{15}N -PDT. An equal volume of each cell preparation was then drawn into a capillary tube, the tube was sealed and introduced in a Bruker EMX EPR spectrometer operating at 9 GHz. EPR spectra were recorded every other minute for linewidth determination. All samples were kept at 37°C during all measurements.

EPR Calibration

The linewidth of ^{15}N -PDT was calibrated at various % O_2 using different mixtures of air and nitrogen with an Aalborg gas mixer. Exact % O_2 was defined using a Servomex oxygen analyzer OA450. Ten microliters of ^{15}N -PDT 2 mM was mixed with medium containing 20% (w/v) dextran and PBS (ratio 1:1) for the control group, or microbeads (0.5 mg cytodex 1/50 μ l PBS) for the cytodex 1 group.

Trypsinization

For the trypsinized B16F10-luc group, 100 μ l of microbeads were rinsed twice with PBS in a microtube and trypsinized with 200 μ l (final volume) of 0.05% Trypsin-EDTA for 10 minutes at 37°C with gentle agitation. After cell harvesting, trypsin was neutralized with 90 μ l of full medium containing 20% (w/v) dextran and 10 μ l of ^{15}N -PDT was added. This solution was then transferred into a capillary tube for EPR measurements. HUVEC trypsinization was performed as follows: 95 μ l of HUVEC-coated microbeads were collected and washed twice with PBS in order to reach a final volume of 45 μ l. Thirty microliters of 0.05% Trypsin-EDTA were added during 5 minutes at 37°C. Trypsin was then

neutralized with 20 μ l of full medium containing 20% (w/v) dextran, and 5 μ l of ^{15}N -PDT was added prior to EPR measurements.

Collagenase Treatment

One hundred microliters of B16F10-luc grown on cytodex 3 were rinsed twice with PBS, treated with 90 μ l of a collagenase type 1 solution (10 mg/ml) (Worthington) during 15 minutes at 37°C with occasional agitation. One hundred microliters of full medium containing 20% (w/v) dextran and 10 μ l of ^{15}N -PDT were subsequently added, and the resulting cell suspension was drawn into a capillary tube for EPR measurements. The procedure used for HUVEC collagenase treatment was the same as that for HUVEC trypsinization except that trypsin was replaced by a collagenase type 1 solution (10 mg/ml) and the cells were incubated during 15 minutes at 37°C.

Cell Death Assay

Cell death after trypsin or collagenase treatment was determined using the trypan blue method. Briefly, treated cells were mixed with a trypan blue solution (Sigma-Aldrich, ratio 10:1). Detached cells and remaining adherent cells on the microbeads were spread onto a 6-well plate. Pictures were acquired using a Zeiss Axiovert S100, from which dead and living cells were counted using the ImageJ software.

Mitochondrial Loss Following Cell Detachment

Potential mitochondrial loss during cell harvesting from cytodex microbeads using 0.05% Trypsin-EDTA and collagenase was determined with the detection of mouse mitochondrial complex IV subunit 1 (COXI) by western blotting. Briefly, aliquots of 100 μ l of B16F10-luc cells loaded onto cytodex 3 microbeads were rinsed twice with PBS and resuspended in 0.05% Trypsin-EDTA or in a collagenase solution (10 mg/ml) for 10 minutes with gentle agitation. After cell detachment, PBS was added and samples were centrifuged (100 g for 5 minutes). Supernatants were discarded and cells were washed with PBS. After a last centrifugation step, samples were resuspended in Laemmli Buffer 2 $\times$ (6.25% (v/v) Tris HCl 1M pH 6.8–6.9, 10% (v/v) glycerol, 2% SDS, 5% (v/v) β -mercaptoethanol, 0.02% bromophenol blue to match a total volume of 200 μ l). As control, cell-coated microbeads were washed several times with PBS and resuspended in 200 μ l Laemmli Buffer 2 $\times$. After denaturation (3 minutes at 100°C), 30 μ l of the samples were loaded in triplicate in a 10% Mini-PROTEAN TGX Precast Gel (Bio-Rad). After electrophoresis, proteins were transferred onto a 0.4 μ m PVDF membrane using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was

then probed with a mouse anti-COXI antibody (Invitrogen, 1/1000) in PBS containing 0.1% Tween 20 and 5% non fat dry milk (PBST/5% milk) for 2 hours at room temperature with continuous agitation. After two washes with PBST, a peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was added (1/10000). Chemiluminescence was visualized using Western lightning plus ECL (PerkinElmer). Bands were quantified using the ImageJ software. β -actin was used as a loading control and probed using a mouse anti- β -actin antibody (Sigma-Aldrich, 1/10000).

ATP Quantification Assay

B16F10-luc cells were grown in 100-mm diameter dishes until they reached confluency. Adherent cells were washed with PBS and received full medium for 1 hour at 37°C/5%CO₂. Then, cells were washed with PBS and collected in 3 ml of cold lysis buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.01% Triton X-100). For the detached groups (trypsin and collagenase), confluent cells were rinsed with PBS, detached using 0.05% Trypsin-EDTA (5 minutes at 37°C/5%CO₂) and neutralized with full medium. At this step, cells were counted using a hemocytometer and viability was assessed with the trypan blue dye. B16F10-luc cells were centrifuged (1000 rpm for 5 minutes) and resuspended in full medium. Detached cells were kept in suspension in an open 15 ml tube in full medium at 37°C/5% CO₂ with gentle agitation. After 1 hour in suspension, cells were centrifuged, counted and resuspended in 3 ml of cold lysis buffer. Intracellular ATP levels were then determined using a bioluminescence-based ATP Determination Kit (Invitrogen) following manufacturer's instructions. Bioluminescence was measured using a SpectraMax M2 microplate reader (Molecular Devices). These experiments were repeated using collagenase instead of trypsin. The same protocol was applied except that B16F10-luc were seeded on BD BioCoat™ collagen I culture dishes (VWR) and cells were harvested using a collagenase type 1 treatment (10–15 minutes at 37°C).

Glucose Uptake and Lactate Production Assays

B16F10-luc cells were grown in 100-mm diameter dishes (trypsin treatment) or collagen 1-coated dishes (collagenase treatment) until they reached confluency. Cells were washed with PBS, detached using trypsin (5 minutes at 37°C) or collagenase (10–15 minutes at 37°C with continuous agitation). After neutralization with full medium, cells were centrifuged (1000 rpm, 5 minutes) and resuspended in 2 ml of full medium. Cells were kept in suspension for 3 hours (collagenase) or 4 hours (trypsin) at 37°C/5% CO₂ with gentle agitation. After incubation, viability was assessed using the trypan blue dye and supernatants were collected. As a control, confluent dishes were rinsed with PBS and replenished with 2 ml of full medium. After 3 hours (collagenase experiment) or 4 hours (trypsin experiment) at 37°C/5%CO₂, supernatants were also collected. All supernatants were next deproteinized using ultra-centrifugation filter tubes with a 10 kDa cutoff (VWR). Glucose and lactate concentrations were measured using a CMA600 enzymatic analyzer (Aurora Borealis) according to manufacturer's instructions. Deproteinized full medium was used to precisely determine starting basal glucose and lactate concentrations.

Statistical Analyses

All results are represented as means $\pm$ standard error of the mean (SEM). For the ¹⁵N PDT calibration curves, linear regressions and comparisons between slopes (Student's *t*-test) were performed. Student's *t*-test was used for OCR, glucose, lactate and ATP comparisons between adherent and detached cells. COXI

expression was analyzed using one way ANOVA. $p < 0.05$ was considered to be statistically significant.

Results

Dextran Microbeads do not Affect Oxygen-sensing by ¹⁵N-PDT in EPR Oximetry

In a first instance and in order to validate the subsequent experiments, we tested whether naked cytodex microbeads introduced a bias in recorded %O₂ values with EPR or not. Calibration curves of ¹⁵N-PDT obtained with and without dextran microbeads were not statistically different (Figure 2), providing a demonstration that the use of cytodex microbeads is compatible with EPR OCR assays.

Cell Detachment Induces a Significant Decrease in the Respiration Rate of Tumor and Endothelial Cells

Our validated EPR setup was exploited to question the importance of cell adhesion/detachment on the respiration rate of tumor and endothelial cells, i.e. two cell types representing major cell subtypes found in tumors. In each case, cell detachment was performed using two different treatments, trypsin or collagenase, in order to assess the general effect of detachment but also specificities inherent to the harvesting method.

Trypsinization of B16F10-luc murine melanoma cells induced a ~60% decrease in the OCR (Figure 3A, $0.58 \pm 0.03\%$ O₂/minute for adherent *versus* $0.24 \pm 0.06\%$ O₂/minute for trypsinized cells). Trypsinization induced a similar although of less amplitude ~40% reduction in the OCR of HUVECs (Figure 4A, $0.81 \pm 0.03\%$ O₂/minute for adherent *versus* $0.48 \pm 0.07\%$ O₂/minute for trypsinized cells). These data indicate that cell adhesion paces the oxidative metabolism of tumor and endothelial cells at a high rate, whereas cell detachment with trypsin induces a metabolic reprogramming towards a less oxidative phenotype. Cell survival was only moderately affected by the treatment, with a 94% B16F10-luc and a 91% HUVEC survival after trypsinization.

Since trypsinization is known to affect the expression of proteins that regulate cell growth, metabolism, adhesion,... [26,27], we took advantage of collagen-coated cytodex 3 microbeads to use collagenase instead of trypsin to achieve cell detachment. The collagenase treatment of B16F10-luc-coated beads allowed efficient cell harvesting (Figure 3F). Also in these smoother

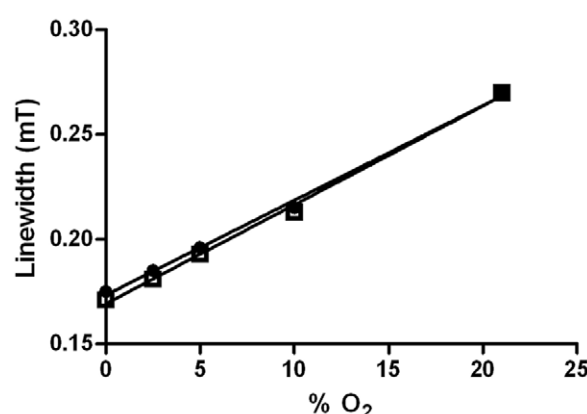


Figure 2. Calibration of ¹⁵N-PDT in function of %O₂. Linear regression of nitroxide alone (●) does not differ from calibration of ¹⁵N-PDT with cytodex 1 beads (□).

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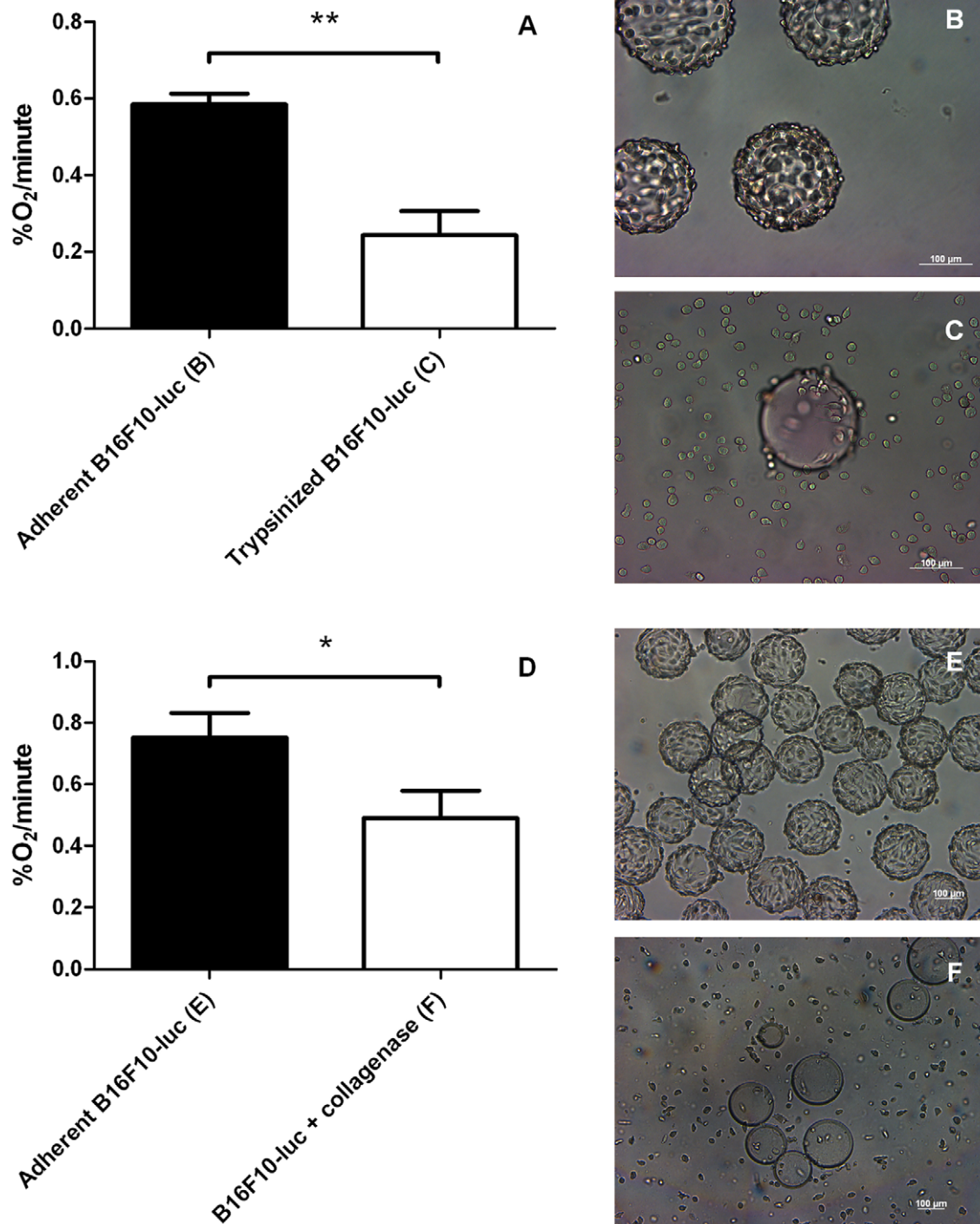


Figure 3. Effect of detachment procedures on B16F10-luc tumor cells. OCR values (%O₂/minute) (A, D) of adherent B16F10-luc and detached B16F10-luc. Trypsinized (n = 3) or collagenase group (n = 4) show a decreased oxygen consumption rate compared to control groups (n = 3 for A, n = 6 for D). Results are statistically significant (***p* < 0.01 for trypsinized cells, **p* < 0.05 for B16F10-luc + collagenase). Pictures of each treatment are displayed next to the graphs. Trypsin treatment (C) or dissolving the coating of Cytodex 3 microbeads (F) result both in cell detachment while control cells (B and E) remain adherent. Data are expressed as means ± SEM. doi:10.1371/journal.pone.0053324.g003

experimental settings, cell detachment accounted for a net reduction in O₂ consumption (Figure 3D, 0.75 ± 0.08% O₂/minute for adherent *versus* 0.49 ± 0.09% O₂/minute for detached cells). It was confirmed with HUVECs (Figure 4D, 0.81 ± 0.03% O₂/minute for adherent *versus* 0.57 ± 0.07% O₂/minute for the

collagenase group). The collagenase treatment was found to be responsible for a less pronounced OCR inhibition (34% for B16F10-luc, 30% for HUVECs) compared to trypsin, while cell viability was totally preserved similarly to trypsin (data not shown). Our data collectively indicate that cell detachment generally

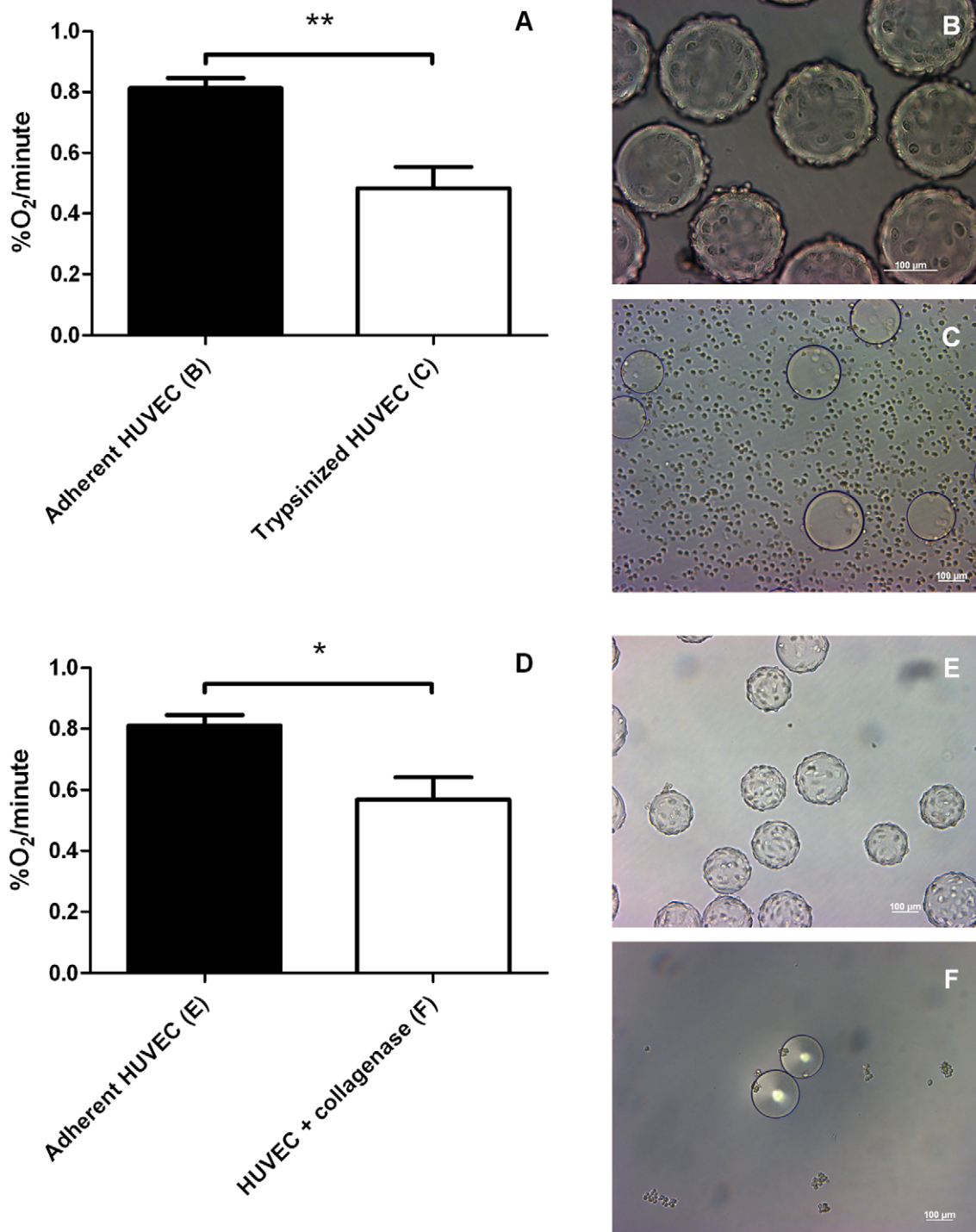


Figure 4. Effect of detachment procedures on HUVEC cells. OCR values (%O₂/minute) (A, D) of adherent and detached HUVECs. Trypsinized (n = 3) or collagenase group (n = 3) show also a decreased oxygen consumption rate compared to adherent group (n = 3 for A and D). Results are statistically significant (***p* < 0.01 for trypsinized cells, **p* < 0.05 for HUVEC + collagenase). Microscopy pictures confirm cell detachment using trypsin (C) and collagenase (F) while control cells remain adherent (B, E). Data are expressed as means ± SEM.
doi:10.1371/journal.pone.0053324.g004

reduces the OCR of tumor and endothelial cells. HUVECs were grown on Cytodex 3 and both harvesting methods were carried out from the same batch of cells, meaning that the same control was used for both treatments. Furthermore, to ensure that the observed decreases in the OCR reflect cellular stresses induced by

detachment procedures and not experimental bias, mitochondrial COXI protein expression was assessed using Western Blotting (Figure 5). COXI expression was not significantly altered when cells were detached with trypsin or collagenase ($100 \pm 7.02\%$

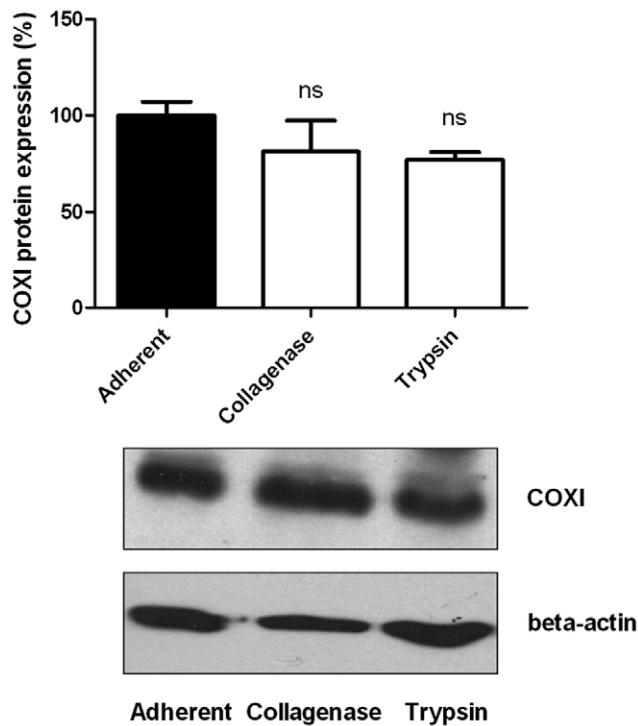


Figure 5. Effect of detachment procedures on COXI protein expression. Trypsinized cells ($n=3$) or collagenase-treated cells ($n=3$) have similar mitochondrial COXI protein levels than adherent cells ($n=3$) (ns, $p>0.05$ (one way ANOVA) compared to adherent cells). Data are expressed as means $\pm$ SEM.
doi:10.1371/journal.pone.0053324.g005

COXI protein expression for attached cells, $81.06 \pm 16.23\%$ for collagenase, $76.63 \pm 4.22\%$ for trypsin).

Cells in Suspension Undergo ATP Depletion, Altered Glucose Metabolism and Significant Cell Death

After having observed that cell detachment impairs mitochondrial respiration, we aimed to check whether keeping cells in suspension could affect their ATP content. As shown in Figure 6, intracellular ATP levels dropped 1 hour post detachment whatever the procedure. (Figure 6A, adherent B16F10-luc: $100.0 \pm 11.94\%$ normalized ATP content, trypsinized B16F10-luc: $28.17 \pm 4.8\%$ normalized ATP content; Figure 6B, adherent B16F10-luc: $100.0 \pm 21.13\%$ normalized ATP content, B16F10-luc+collagenase: $14.64 \pm 3.87\%$ normalized ATP content). Trypan blue viability tests performed 1 hour after detachment (trypsin and collagenase) did not show any significant cell death (data not shown).

Because mitochondrial activity was perturbed after a detachment procedure and because cells in suspension had lower amounts of intracellular ATP, we tested whether other main metabolic pathways were also perturbed. Glucose and lactate concentrations were measured after 3 hours (collagenase group) or 4 hours (trypsin group) after detachment. B16F10-luc in suspension after a trypsin treatment (Figure 7) consumed significantly less glucose (Figure 7A, adherent B16F10-luc: $100.0 \pm 3.03\%$ normalized glucose uptake, trypsinized B16F10-luc: $60.38 \pm 4.01\%$ normalized glucose uptake) but generated similar amounts of lactate compared with attached cells (Figure 7B, 100.0 ± 7.73 and $103.0 \pm 3.30\%$ normalized lactate production for adherent B16F10-luc and trypsinized cells respectively). The experiment was repeated using collagenase instead of trypsin (Figure 8). It

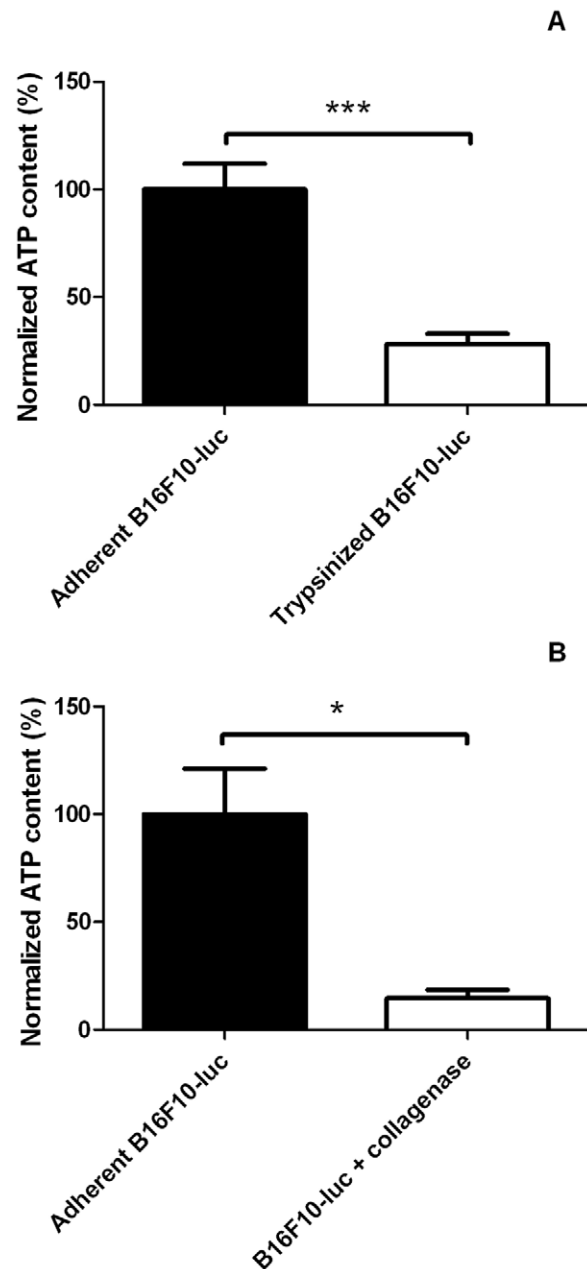


Figure 6. Influence of cell detachment on the intracellular ATP content of B16F10-luc. (A) Trypsinized cells ($n=6$) display a marked ATP depletion compared to adherent cells ($n=6$). (B) The same experiment was carried out using collagenase to harvest cells attached on collagen-coated plates ($n=3$ for control and B16F10+ collagenase groups) and led to similar conclusions. Results are statistically significant ($***p<0.001$ for trypsinized cells, $*p<0.05$ for B16F10-luc+collagenase). Data are expressed as means $\pm$ SEM.
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showed in this instance that detached cells consumed similar levels of glucose (Figure 8A, $100.0 \pm 3.49\%$ normalized glucose uptake for adherent group, $95.51 \pm 3.00\%$ for detached group) but released more important amounts of lactate (Figure 8B, $100.0 \pm 7.46\%$ versus $174.4 \pm 9.33\%$ normalized lactate production for adherent B16F10-luc and B16F10-luc+collagenase respectively) compared with adherent cells. When considering the lactate production/glucose consumption ratio (glycolytic index), both

harvesting methods led to an increased glycolytic index (Figure 7C for trypsin experiments, glycolytic index = 1.73 ± 0.14 for adherent cells, 2.98 ± 0.26 for trypsinized cells; Figure 8C for collagenase experiment, glycolytic index = 0.89 ± 0.39 for adherent cells *versus* 1.625 ± 0.36 for detached cells). Significant cell death was observed at later time points after cell detachment (Figure 8D, $63.91 \pm 1.38\%$ survival in collagenase group; Figure 7D, $79.71 \pm 1.54\%$ survival in trypsin group).

Discussion

This *in vitro* study shows that detached cells consume highly significantly less oxygen than adherent cells, implying that cell adhesion promotes cell respiration and cell detachment protocols

mitochondrial uncoupling. OCR inhibition appeared quickly after harvesting when viability was preserved. However, cells remaining in suspension had decreased intracellular ATP levels, which is in accordance with previously published results [28]. Although this net reduction in intracellular ATP is coherent with a decreased OCR, we cannot exclude that detached cells consume ATP much faster than adherent cells in order to maintain cellular homeostasis. We further observed that cells in suspension after both trypsin and collagenase treatments for a prolonged period (3–4 hours) exhibited a higher glycolytic index, indicating that other nutrients than glucose (such as glutamine which was present in the experimental medium) became a significant source of lactate when cells are detached. Eventually, a significant proportion of cells did not survive when kept in suspension for longer time.

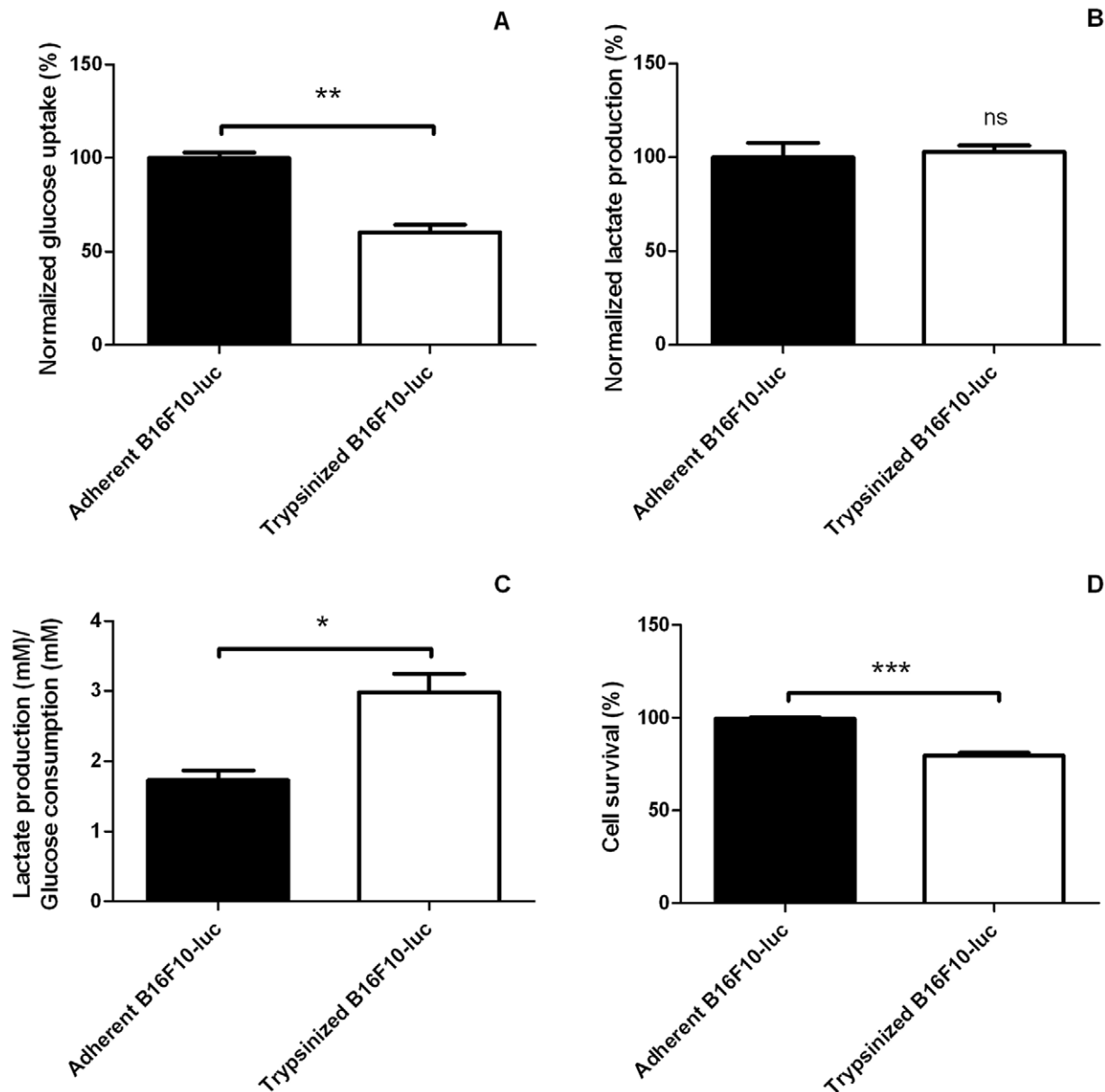


Figure 7. Glucose metabolism in adherent and trypsinized B16F10-luc. Trypsinized B16F10-luc ($n=3$) take up less glucose (A) and release similar amounts of lactate (B) than adherent cells ($n=3$). Cell detachment therefore accounts for an increased lactate production/glucose uptake ratio (C). Prolonged detachment (4 hours) affects cell survival (D). Results are statistically significant (** $p<0.01$ for A, ns, $p>0.05$ for B, * $p<0.05$ for C, *** $p<0.001$ for D). Data are expressed as means $\pm$ SEM.
doi:10.1371/journal.pone.0053324.g007

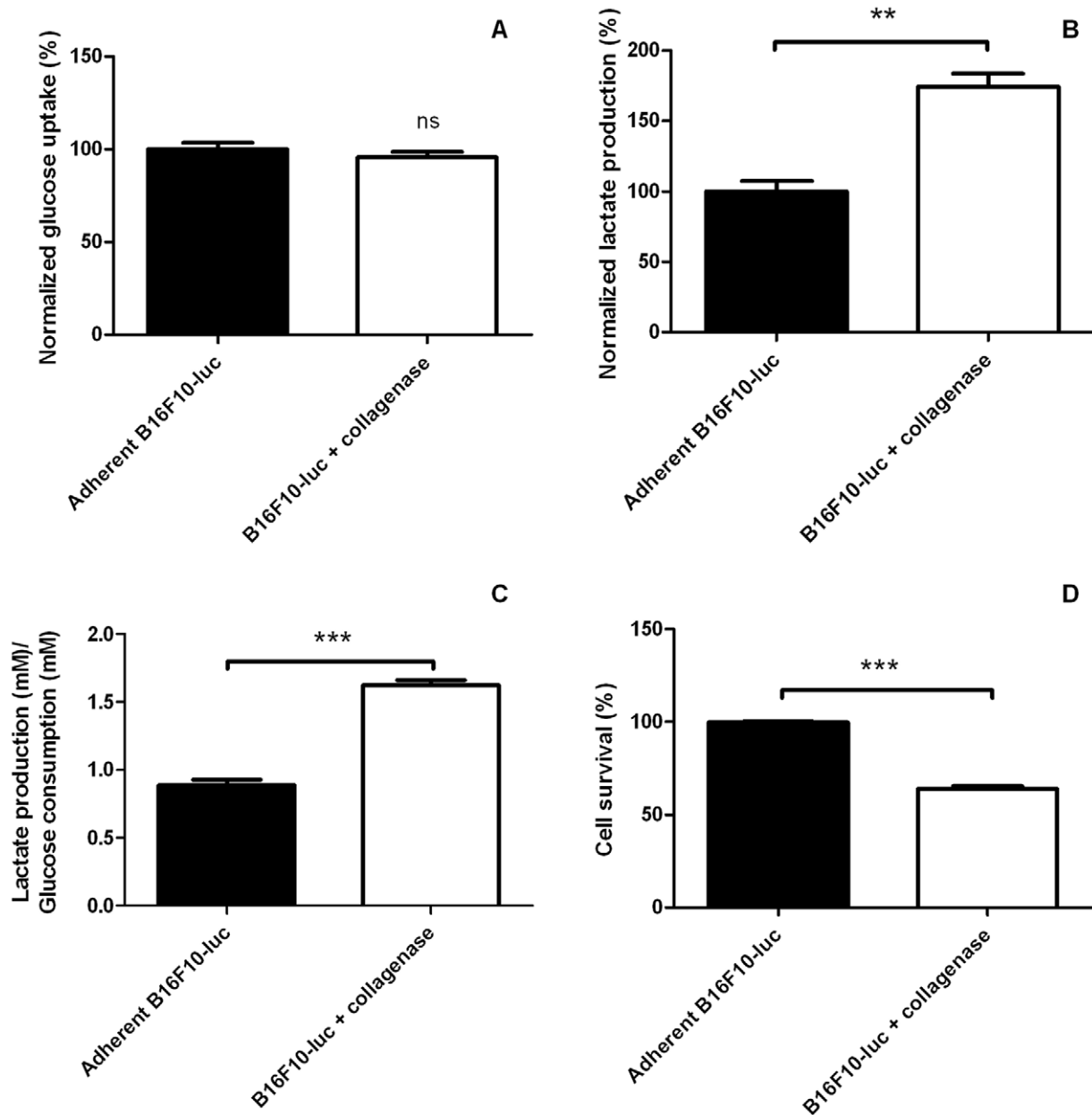


Figure 8. Glucose metabolism in adherent and B16F10-luc detached using collagenase. B16F10-luc seeded on collagen-coated plates and harvested using collagenase ($n = 3$) take up similar levels of glucose (A) but release more important amounts of lactate (B) than adherent cells ($n = 3$). Cell detachment results thus in an increased lactate production/glucose uptake ratio (C). Prolonged detachment (3 hours) affects cell survival (D). Results are statistically significant (ns, $p > 0.05$ for A, $**p < 0.01$ for B, $***p < 0.001$ for C, $***p < 0.001$ for D). Data are expressed as means $\pm$ SEM. doi:10.1371/journal.pone.0053324.g008

Surprisingly, survival was better for trypsin-treated cells compared to collagenase-treated cells. A reasonable explanation is that for this specific experiment, on the one hand trypsin exposure was much shorter and on the other hand vigorous pipetting was necessary to detach cells adherent to a collagen substrate when using collagenase. Altogether, we evidenced that detachment affects several key metabolic parameters.

Although other reports have already stated that mechanically detached cells or trypsinized cells have decreased metabolic activities (decreased glucose oxidation and oxygen consumption) [29,30], our observations extend the paradigm to the less aggressive harvesting collagenase protocol and thereby show that

numerous commonly used detachment methods may disturb mitochondrial respiration. Nevertheless, our study highlights that prolonged cell suspension is followed by important metabolic perturbations (glucose metabolism, intracellular ATP, ...). There is therefore strong evidence that preserving cell adherence is a preferred condition for accurate metabolic studies.

Interestingly, for both B16F10-luc cells and HUVECs, OCR inhibition was more pronounced using trypsin than collagenase. Since trypsin acts through cleaving membrane proteins and collagenase is essentially a protease of the extracellular matrix, OCR inhibition upon detachment would involve not only the physical cell-surface and cell-cell interactions but also more

generally signals originating from the cell membrane, involving anchor proteins or not, and influencing cell metabolism. Metabolic adaptations to cell detachment and loss of cell-matrix contacts have previously been studied in different setups. Accordingly, it is known that cells losing contacts with their surrounding mainly *via* integrin signalization produce an increased level of reactive oxygen species (ROS), activate pro-apoptotic cascades, and eventually undergo a specific form of cell death termed anoikis [31,32]. With a focus set on cell metabolism, it has been shown that detached cells have decreased glucose uptake rates affecting both glycolysis and the pentose phosphate pathway, and that oxidative stress following detachment impairs fatty acid oxidation [33–35]. Our observations are thus in line with these previous studies albeit using an innovative, sensitive and reproducible EPR approach allowing straightforward *in vitro* oxygen measurements.

Although the deregulation of major metabolic pathways is a pertinent cause of OCR inhibition, other mechanisms are probably involved. The existence of a transmembrane electron transport chain consuming oxygen to maintain the cellular redox status within a physiological range has been shown to be a significant contributor to global cell O₂ consumption in addition to mitochondrial respiration [36]. Thus, altering the membrane organization with detachment might strongly affect these enzymatic processes. It could explain why trypsin, cleaving non specifically membrane enzymes, has a more important impact on OCR than collagenase for both studied cell lines (approximately 50% inhibition for trypsin and 30% for collagenase treatments). Cell detachment is also naturally followed by deep morphological

changes. Harvested cells exhibit spherical shapes which differ from the adherent phenotype, implying that the actin cytoskeleton architecture and outside-in signaling would logically be altered following detachment procedures. Since many glycolytic enzymes are localized in the cytoplasm and sequestered near actin filaments, their activities could be affected by such morphological alterations [37].

On a methodological point of view, the use of microcarrier technology and EPR oximetry combines the advantages of both techniques. Although oximetry methods on adherent cells have been described (such as with the Seahorse approach) [21,38,39] and cytodex beads have already been used in combination with Clark electrodes [40], our technology provides the unique advantage to be highly sensitive so that it can theoretically detect even small OCR changes likely due to biologically significant variations, whereas the stressful steps used here for proof-of-principle validation and with other techniques are relevant for sample preparation. Therefore, implementation of this new protocol could be a promising tool for further metabolic cell characterizations.

Author Contributions

Conceived and designed the experiments: PD BFJ PS BG. Wrote the paper: PD TC GDP PL OF BFJ PS BG. Analyzed the data: PD GDP BFJ PS BG. Performed the experiments: PD TC GDP PL. Contributed reagents/materials/analysis tools: PD TC GDP PL OF BFJ PS BG.

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Chapter IV: Discussion

I. Main findings

The aim of the thesis was to use MRI cell tracking for monitoring the fate of metastatic (U)SPIO-labeled cancer cells using experimental and spontaneous models of metastasis.

First, we took advantage of X-band EPR for assessing the iron oxide content in cells after passive labeling with (U)SPIO (Danhier 2012). It was shown that EPR was reliable, sensitive and specific for this purpose.

Second, we used a multimodal approach, including BLI and MRI, for tracking USPIO-labeled murine renal cancer cells after intrasplenic injection (experimental metastased to the liver) or intrarenal injection (spontaneous metastases to the lungs and lymph nodes) (Danhier 2013b). Several limitations of MRI cell tracking were highlighted, such as the quick loss of the label after injection and the impossibility to visualize USPIO-labeled cells in the lungs and in the liver. These organs however, are common metastatic sites, calling for the development of other imaging strategies. The use of *ex vivo* EPR for quantifying the iron oxide content in mouse tissues allowed the short-term assessment of the spread of labeled cancer cells after injection. Furthermore, BLI and anatomical liver MR images were shown to assess accurately the late colonization of organs by cancer cells.

Third, it was aimed to image the homing of breast cancer cells to the mouse brain after intracardiac injection (experimental metastasis). In contrast with the previous part of the study (Danhier 2013b), MRI was shown to be sensitive for the detection of USPIO-labeled breast cancer cells on T_2^* -weighted images (Danhier 2013c). In addition, it was possible to accurately quantify *ex vivo* the number of labeled cells entrapped in the brain shortly after injection using X-band EPR. As in the previous part of the study (Danhier 2013b), a drop in the iron oxide content together with a loss of contrast on MR images were observed one day after delivery (Danhier 2013c). This part of the study showed that magnetic labeling approaches (combining EPR and MRI) hold promises in preclinical models for assessing brain metastasis development from the initial entrapment of cancer cells.

Last, we investigated the impact of cell detachment on cell metabolism (Danhier 2013a). Using two different harvesting protocols, we showed that both cancer and endothelial cells underwent an important metabolic stress after detachment. Using EPR oximetry, it was shown that mitochondrial respiration was severely impaired upon detachment. At the same time, the ATP content was decreased and the glycolytic rate was increased, suggesting a glycolytic compensation for OXPHOS defect.

II. Considerations and perspectives

II.1. *Ex vivo* labeling of cancer cells

The first major hurdle encountered in this work was the magnetic labeling of cancer cells. Indeed, an efficient *ex vivo* magnetic labeling was mandatory to ensure the validity of subsequent *in vivo* experiments. First, many iron oxides (e.g., Ferumoxides and MPIO) used in preliminary studies remained attached to the plasma membrane of the cells or clustered outside the cells. In particular, MPIO, used in most cancer cell tracking studies (Heyn 2006, Foster 2008, Townson 2009, Ribot 2011), were found to be poorly internalized by the cell lines used in this work (Figure 24), whereas USPIO Molday Ion rhodamine B[®] or MIRB (colloidal size of 35 nm) used in this study were shown to be optimal for labeling cancer cells. These results are in direct contradiction with precedent studies that indicated that SPIO were more efficiently internalized than USPIO (Sun 2005, Oude Engberink 2007). The discrepancy between the literature and the present results might be due to the number of variables (cell density, type of particle, culture medium, cell type) affecting the yield of labeling. These observations lead to the conclusion that labeling efficiency (pg Fe/cell) and label localization must always be tested prior to a MRI cell tracking experiment. However, knowing the mean intracellular iron oxide content of a cell population does not give information on the distribution of the label among the cells. In our experiments, fluorescence microscopy showed that 100 % of cells were labeled after incubation with MIRB.

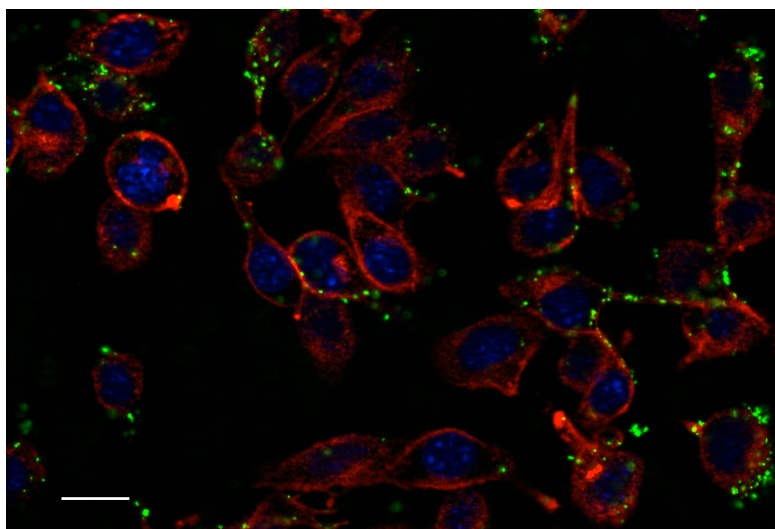


Figure 24: B16F10-luc melanoma cells after an overnight incubation with MPIO conjugated with the fluorophore Green Dragon (green fluorescence). Most of MPIO particles remain adherent on the plasma membrane. Nuclei are stained in blue using Hoechst 33342 and plasma membranes are stained in red using wheat germ agglutinin-rhodamine. The scale bar represents 20 μm .

Second, it was challenging to verify in a quantitative and reproducible manner the efficiency of the labeling procedures. In the first part of the study (Danhier 2012), limitations of (fluorescence) microscopy were evidenced for such purposes. In this context, the use of EPR allowed a simple and reproducible assessment of the iron oxide content in cells (Danhier 2012). The sensitivity and the low volumes used in EPR experiments further allowed sparing fragile or rare cells (human stem cells, primary cells). Therefore, this approach would be particularly useful for other applications such as the optimization of cell tracking procedures for cell-based therapies. At room temperature, EPR measures superparamagnetic forms of iron, whereas ICP-MS measures elemental iron. After an overnight incubation of USPIO, an agreement between intracellular iron oxide contents measured with EPR and ICP-MS was observed (Danhier 2012). It would have been interesting for the subsequent studies to repeat these experiments at later time points to investigate the metabolism of iron oxide nanoparticles once ingested by cells. Arbab *et al.* used a similar approach for studying the dissolution of iron oxides in lysosomal environments (Arbab 2005). In this study, relaxometry measurements and spectrophotometry were used for assessing the evolution of MR signal as well as the levels of Fe^{3+} . The iron oxide quantification using EPR is based on the signal intensity, measured by double integration, of the EPR spectrum of iron oxides. In addition to intensity, the shape of the EPR spectrum of USPIO was affected after internalization as shown in Figure 25. The exact reasons for these changes (that may be related to the clustering of USPIO within endosomes, metabolism, or other yet unknown parameters), as well as the possible implications on MR relaxivities, certainly warrant further investigation.

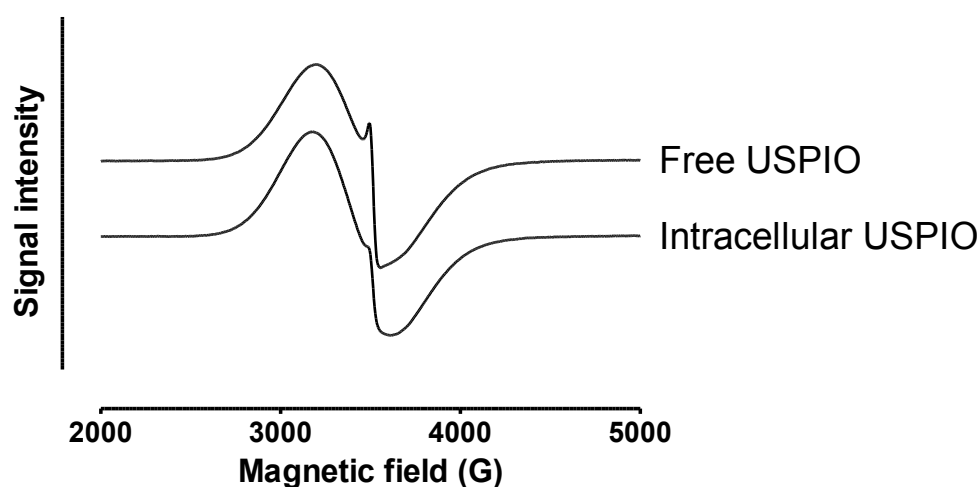


Figure 25: Influence of cellular internalization on the EPR spectrum of iron oxides (USPIO MIRB).

We also showed that serum deprivation led to a higher yield of labeling for two cancer cell lines (Danhier 2012). This can be due to an inhibition of proliferation, modifications of the physiochemical properties of USPIO suspension (protein corona) or changes of the endocytosis behavior of the cells. Owing to the elevated proliferation rate of cancer cells, dilution of the USPIO content among daughter cells during labeling may be the most important factor explaining the effect of serum deprivation on iron oxide incorporation (Treuel 2013). Serum deprivation was chosen in the second part of the study for labeling luciferase-expressing RENCA-luc renal adenocarcinoma cells in order to optimize visualization of MIRB-labeled cells *in vivo* (Danhier 2013b). It must however be pointed out that serum deprivation also induces autophagy and oxidative stress (Dyllick-Brenzinger 2010). Moreover, it was shown that R_2 values of samples containing iron oxide-labeled fibroblasts reached a plateau after a defined level of internalized iron oxides (Boutry 2008). Therefore, performing relaxometry experiments or T_2 maps of MIRB-labeled cell suspensions may be relevant to investigate the need of serum deprivation for labeling cells prior to injection and imaging.

II.2. Potential use of EPR for monitoring the expression of MR reporter genes

Using low temperatures, EPR allows the study of different endogenous iron forms including transferrin and ferritin (Krzyminiwski 2011). Therefore, EPR spectroscopy could be useful for assessing the expression of iron-based reporter genes. As mentioned in the introduction section of this work, tyrosinase is the main enzyme involved in the synthesis of melanin and has been proposed as a MR reporter gene (Gilad 2007b). The natural production of paramagnetic melanin in melanomas constitute an attractive approach to monitor metastasis, and EPR may be used to determine the melanin content of organs in which melanoma metastases settle and accumulate. Mouse B16 melanoma is one of the most studied cell lines in metastasis research. Different cell subtypes have been characterized and possess different metastasis patterns. For instance, B16F10 melanoma cells are highly metastatic to the lungs (Fidler 1973), whereas the variant subline B16-F10-B2 produces brain metastasis (Raz 1980). Because the EPR signal intensity directly correlates with the number of melanoma cells, *ex vivo* X-band EPR spectroscopy constitutes a unique tool to monitor melanoma progression to complement MRI observations (Godechal 2011). This would allow the quantitative assessment of the efficacy of new anti-metastatic treatments. A prerequisite is

however that the melanin content per cell remains constant during the course of the metastatic process.

II.3. MR imaging of (U)SPIO-labeled cancer cells

Once an efficient labeling procedure was found (Danhier 2012), it was next aimed to visualize disseminated MIRB-labeled cancer cells at distant sites away from a primary tumor. Visualizing (U)SPIO-labeled cells at the injection site was quite straightforward (Danhier 2013b). On the other hand, the presence of confounding factors such as blood vessels, hemorrhages and air-tissue interfaces hampered the visualization of labeled cells at distant sites. Experiments that we performed in this study showed that gradient-echo images (T_2^*) were necessary for detecting isolated MIRB-labeled cells (Danhier 2013c). Smirnov *et al.* previously showed that SPIO-labeled cells were barely visible on spin echo sequences (T_2 -weighted), whereas detection was greatly improved using gradient-echo sequences (T_2^* -weighted) at 9.4 T (Smirnov 2006). Besides acquisition parameters, the ability to discriminate (U)SPIO-labeled cells on T_2^* -weighted images from surrounding tissues depends on the intensity of the signal void and on the presence of intrinsic areas of signal loss in the tissue. First, the darkening effect strongly depends on the type of particles as well as on the local iron oxide concentration. As stated before, signal voids induced by MPIO are much more important than that of USPIO (Shapiro 2008). However, MPIO were not chosen as labeling agents because of their poor internalization profile. The amount of labeled cells in a given volume is also critical. It was obviously easier to observe a large number of MIRB-labeled cancer cells in the kidney than isolated metastatic cells in the mouse brain (Danhier 2013b, Danhier 2013c).

Second, all tissues are not “equivalent” with respect to MR imaging (U)SPIO-labeled cells. Visualizing (U)SPIO-labeled cells in the lungs was not possible using conventional T_2 -, T_2^* -weighted images because lungs are devoid of proton signal and contain many air-tissue interfaces (Bulte 2011). It was shown that T_2^* of lung tissues was lower than 0.8 ms for small animals at 2 T (Kuethe 2007). Detecting SPIO in lung tissues at 11.7 T was thus impossible owing to these ultra short relaxation times. Imaging SPIO-labeled cells in the lungs would require the use of hyperpolarized ^3He MRI (Branca 2010). Liver imaging was also difficult owing to the presence of movements and because of the heterogeneous appearance of the liver tissue on T_2 -, T_2^* -weighted images (Danhier 2013b). In this regard, working at high field (11.7 T) was perhaps not optimal: it improves the signal to noise ratio and by consequence the contrast, but T_2^* susceptibility artifacts are also increased and might impair the detection of

cells of interest. Moreover, the darkening effect due to the presence of high levels of endogenous paramagnetic iron species (e.g., ferritin) in iron-rich organs such as the liver is also increased with high external magnetic fields (Ziv 2010). Concerning liver images displayed in this work (Danhier 2013b), it can be speculated that signal voids due to labeled cancer cells were masked by the presence of numerous susceptibility artifacts (Figure 26). Hence, 11.7 T MRI did not allow to ascertain the presence of metastatic MIRB-labeled cancer cells. EPR measurements performed one day after injection and the presence of macrometastases shown by MRI and BLI a few weeks after injection confirmed that metastatic cells were actually present in the liver shortly after injection. As shown in Figure 26, the fate of iron oxide-labeled hepatocytes in the liver was achieved using MPIO as labeling agent at 1.5 T. At lower magnetic field, signal arising from liver parenchyma appears more “homogenous” on T_2^* -weighted images than at 11.7 T (Shapiro 2006, Leconte 2010). Hence, an equilibrium between magnetic field strength, signal to noise ratio and the apparition of intrinsic signal voids must be found to efficiently visualize iron oxide-labeled cells.

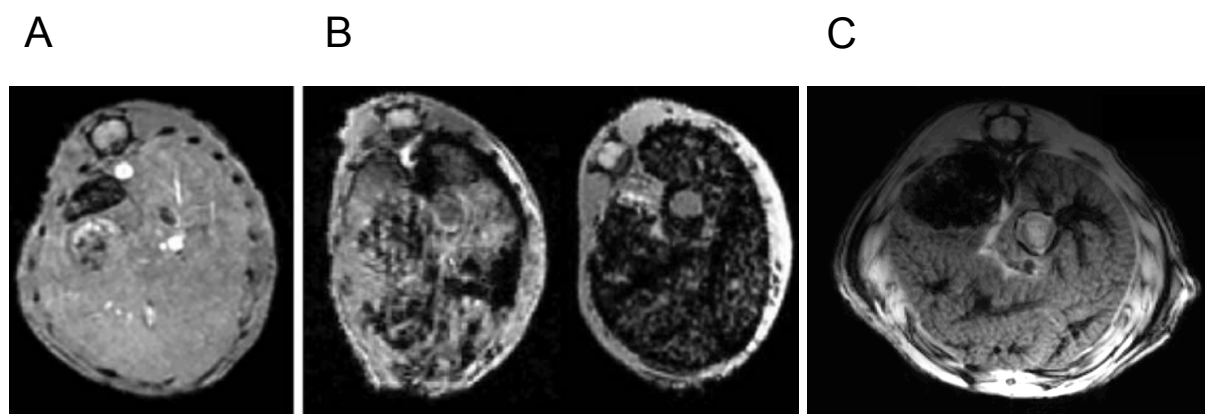


Figure 26: Comparison between T_2^* -weighted images of the mouse liver at 1.5 T and 11.7 T. (A) T_2^* -weighted image of a control liver and of a liver transplanted with hepatocytes labeled with MPIO (10^6 cells/mouse using intrasplenic injection) (B) (Leconte 2010). (C) T_2^* -weighted image of the mouse liver at 11.7 T (extracted from Danhier 2103b). Of note, the liver appears more homogenous at 1.5 T (A) than at 11.7 T (C).

On the other hand, imaging (U)SPIO-labeled cells in the mouse brain was easier (Danhier 2013c). In particular, the use of a cryoprobe improved significantly the signal to noise ratio. Moreover, cerebral tissues appeared hyperintense and homogenous on T_2^* -weighted images. The presence of susceptibility artifacts close to ear cavities limited the detection of MIRB-labeled cells in these regions. Specific imaging sequences, such as gradient-echo slice excitation profile (GESEPI), were designed to overcome this problem (Yang 1999, Demeure 2002). These observations illustrate that “hot spot” imaging methods

(e.g., BLI, PET scan), allow an easier data interpretation when the signal source is known and when the endogenous “parasitic” signal is very low.

Several strategies exist to overcome these issues. First, the development of “positive contrast” techniques allowing to detect SPIO-labeled cells as hyperintense spots is promising. However, anatomical background information is lost with these techniques and “positive contrast” images must be overlaid to anatomical MR images (Liu 2009). Second, magnetic particle imaging (MPI) is an emerging technique that allows direct measurements of iron oxide particles in living organisms. The main advantages of MPI are the lack of confounding endogenous contrast sources and quantification of the iron oxide content (Bulte 2011, Borgert 2012). However, MPI equipments are still prototypic and need further developments in order to improve the detection threshold ($\sim 10^4$ SPIO-labeled cells) (Saritas 2013).

II.4. *Ex vivo* iron oxide quantification for SPIO-based cell tracking studies

The quantification of the number of cells is important for cell tracking studies. As discussed in the third part of our study (Danhier 2013c), *in vivo* iron oxide quantification using MRI is challenging. Hence, EPR was found to be a complementary tool to MRI for cell tracking studies. A good agreement was found between signal voids on T_2 -, T_2^* - weighted images and the iron oxide content measured in kidney and brain samples. With regard to *ex vivo* iron quantification, we focused on measuring superparamagnetic forms of iron. Iron oxide biodistribution studies performed after intrasplenic or intrarenal injection of MIRB-labeled RENCA cells strongly suggested that MIRB nanoparticles were quickly degraded and cleared after injection (Danhier 2013b). The degradation of iron oxides was found to be slower when free USPIO were injected compared to USPIO-labeled cells. Macrophages, often present at tumor sites, may take up iron oxide particles released by dead cells and degrade SPIO (Shih 2011). Histological studies aimed to check the presence of macrophages would confirm this hypothesis. The dilution effect showed *in vitro* in the second part of the study (Danhier 2013b), explains the signal loss on the MR image but not the drop of iron oxide content in organs. In addition to the important dilution effect in direct cancer cell tracking studies, biotransformation of SPIO was found to be an additional limiting factor for imaging the fate of cancer cells. For assessing the homing of brain metastases, it was found that both iron oxide content and MR signal decreased with time after injection. The fact that USPIO used in these studies may be metabolized complicates the interpretation of these data. With inert particles, the drop in iron oxide content would have unambiguously reflected the

elimination of cancer cells from the mouse brain after initial entrapment. This last information is important to confirm the homing of cancer cells in the brain.

Concerning the fate of MIRB particles *in vivo*, our data (Danhier 2013c) differ from one study led by Cromer Berman *et al.*, who monitored the fate of neural stem cells labeled with MIRB after transplantation in the brain of immunodeficient graft-accepting mice or immunocompetent graft-rejecting mice (Berman 2011). Surprisingly, they observed that contrast persisted in immunocompetent mice for long periods of time (90 days), whereas contrast decreased in immunodeficient mice. It is reported that most immune cells crossing the blood brain barrier and activated in the CNS undergo apoptosis. In immunocompetent mice, it was suggested that immune cells that internalized USPIO released from dead neural stem cells eventually died, whereas the dilution effect was proposed to explain the signal loss on MR images in immunodeficient mice. It therefore seems that biological processes involved the Berman study and the results shown in the present work are different. However, our observations are in line with the study of Faucher *et al.*, discussed in the introduction (Figure 7) (Faucher 2012). After injection in the brain parenchyma of MIRB-labeled glioma cells, authors observed a mismatch between the tumor area and the signal void induced by USPIO after seven days. A loss of contrast with time was also reported 14 days after implantation. Differences in kinetics of MIRB clearance from the tumor between our study and the work of Faucher *et al.* may be due to differences between the renal and brain microenvironments as well as to the nature of cell lines used. To conclude, combining *ex vivo* EPR, ICP-MS measurements and histological studies would be mandatory for thoroughly assessing the fate and metabolism of iron oxide nanoparticles after administration (Levy 2011). Moreover, ensuring the *in vivo* stability of iron oxide nanoparticles is revealed to be necessary even for short-term cell tracking studies.

II.5. MRI cell tracking studies for assessing cancer metastasis

Cancer cell tracking applications allow the qualitative assessment of dormant and proliferating metastases (Townson 2009). Surprisingly, metastatic RENCA cells were found in the lungs shortly after intrarenal injection, meaning that this “spontaneous” model of metastasis is actually an “experimental” model (Danhier 2013b). The alternative 4T1 breast cancer model used in our study did not produce macrometastases in the brain, whereas RENCA did produce macrometastasis but isolated cells could not be visualized using MRI. The use of brain seeking variant cell lines producing brain macrometastases, such as 231-BR

derived from the MDA-MB-231 human breast cancer cell line, would be necessary for studying the short-term fate of CTCs using magnetic cell tracking (EPR and MRI) and the subsequent colonization of the brain parenchyma using conventional MRI (Perera 2012). Because it was possible still to assess the number of cells in the brain shortly after injection, it would be feasible to determine a “metastatic colonization rate”, i.e., the ratio between the number of entrapped CTCs and subsequent macrometastases. In this regard, the ability to accurately quantify the aggressiveness of metastatic cancer cells constitutes the most promising perspective of this work. Many studies focused on cancer metastasis rely on the use of BLI for assessing metastatic burden (Zhang 2009, Daenen 2011). BLI mainly reflects tumor growth and does not provide information on the number, the accurate localization and the size of metastases (Danhier 2013b). On the other hand, our magnetic labeling approach, implemented for tracking brain metastases, allows to monitor metastases directly after the entrapment of cancer cells in the brain. Moreover, this technique could be combined with functional imaging, e.g., magnetic resonance spectroscopy and/or diffusion MRI, to provide information on the metabolic and viability status of macrometastases. As aforementioned, the preferred model for such studies is a metastatic variant of the MDA-MB-231 human breast cancer cell line. Indeed, the validity of this cell line as a model has been confirmed with MOBILE (mapping of oxygen by imaging lipids relaxation enhancement), a MR-related oxymetry technique that relies on the increase of R_1 in fat tissues when the lipid oxygenation is improved (Jordan 2012). Because hypoxia is a driving force of aggressiveness and metastatic progression (Avni 2011, Semenza 2012), combining iron oxide-based cell tracking with MOBILE is now warranted to better characterize microenvironmental regulations of brain metastases.

Our study highlighted that magnetic cell tracking allows the detection (MRI) and quantification (EPR) of CTCs at distant sites from a primary tumor (Danhier 2013b, Danhier 2013c). Is this information relevant for understanding metastasis and for validating new treatments? On the one hand, the magnetic labeling strategy is compatible with indirect approaches such as BLI, and it allows to dissect the metastatic cascade. As stated before, most metastasis studies rely on visual inspection or on the use of BLI alone for assessing macrometastasis. Consequently, only tumor growth is tracked. Thus additional direct cell tracking strategies can provide further additional information on the initial entrapment and adhesion of CTCs in distant organs. In this regard, repeating the brain metastasis experiments with untransformed cells would allow to test whether the entrapment of mouse breast cancer cells in the mouse brain is a passive phenomenon or whether it involves some specific adhesion mechanisms (Al-Mehdi 2000). On the other hand, several anti-metastatic treatments

targeting the adhesion of metastatic cancer cells were investigated by others. For example, antiaggregant and anticoagulant drugs were studied for their anti-metastatic activity (Gil-Bernabe 2013). However, an important proportion of cancer patients already harbor CTCs and DTCs at the time of diagnosis (Braun 2005). Thus, anti-metastatic drugs should be aimed to target established metastases: focusing only on the initial entrapment of CTCs at distant sites from primary tumors is not pertinent for the translational validation of new anti-metastatic treatments (Valastyan 2011).

Both linear and parallel progression models of metastasis describe the metastatic process as a sequential phenomenon. However, recent evidences showed using BLI and fluorescence microscopy that some CTCs shed from metastatic lesions are able to repopulate the primary tumor (Kim 2009). This paradigm, known as tumor self-seeding is described to be important for the metastasis process of several cancer types including breast cancer (Kim 2009, Comen 2011). The self-seeding capacity of tumors could not be observed using our direct cell tracking strategies. However, assessing the capacity of SPIO-labeled CTCs to seed into a primary tumor site could be particularly relevant. Of note, the expression of the GFP or the luciferase gene would be mandatory to document the survival of these “foreign” cells within the primary tumor. Such study would be highly relevant to confirm that the primary tumor is a hospitable niche for cells derived from its own metastases.

II.6. Assessing the function/survival of CTCs

The last part of our study highlighted the metabolic changes occurring after a sudden detachment of cancer cells or endothelial cells (Danhier 2013a). Detached cells were shown to have a decreased O₂ respiration rate, increased glycolysis, and underwent a marked ATP depletion. We used both trypsin and collagenase as detaching agents. Trypsin is a serine protease that nonspecifically cleaves peptides at arginine and lysine residues (Olsen 2004). Although this enzyme is routinely used in cell culture, prolonged exposure of cells to trypsin may harm them by cleaving membrane proteins such as growth factor receptors (Huang 2010). On the other hand, collagenase was used to dissolve the collagen coating of Cytodex 3[®] dextran microbeads, and therefore to avoid chemical stresses. Despite these precautions, it cannot be excluded that mechanical forces, notably stirring, did not affect the observed oxygen consumption rate of detached cells.

The impact of cell detachment on cell survival is well documented, whereas studies focused on the metabolic consequences of cell detachment are underrepresented. Reports on

metabolic changes in breast cancer cells induced by detachment led by the group of Brugge *et al.*, relied on the use of 3D culture methods mimicking the extracellular matrix of the mammary gland (Schafer 2009b, Grassian 2011), whereas the *in vitro* model used in our work likely reflected the “life in transit” step occurring when metastatic cells leave the primary tumor (Danhier 2013a). As most CTCs do not survive once shed in the blood circulation, it is now necessary to investigate whether the decrease in metabolic activity observed after cell detachment is reversible or not. To address this question, it would be important to test the recovery of metabolic activities upon reattachment. In this regard, the activation of the AMP-activated protein kinase (AMPK) pathway for overcoming metabolic stress could be of primary importance (Jeon 2012). AMPK is an energy sensor protein activated by low energy state, e.g., a high AMP/ATP ratio. During starvation, AMPK shuts down anabolic pathways and stimulates catabolic pathways, thereby participating in the preservation of the ATP levels in cells (Viollet 2009). Using fluorescent probes, ROS levels were also shown to be increased after detachment, impairing energy production via a blockade of fatty acid oxidation (Li 1999, Schafer 2009b). Using EPR spin trapping experiments for documenting the involvement of ROS in cell detachment would be relevant. Last, glucose and lactate measurements performed after both trypsin and collagenase treatments showed that the glycolytic index, i.e., the ratio between the lactate produced and the glucose consumed, was increased after detachment (Danhier 2013a). However, lactate production was unchanged after a mere trypsin treatment, whereas it was significantly increased after treatment with collagenase. Glucose levels were also shown to be different between trypsin and collagenase treatments. Investigating the role of other nutrient sources such as glutamine should be performed to explain these observations.

Cell detachment is an important feature associated to cancer invasion. But methodologies used in this study could also be useful for cell-based therapies. In addition of assessing the fate of stem cells after injection using cell tracking modalities, isolating and injecting viable cells to the patient is an important challenge. In this regard, mitochondrial respiration is considered as a viability marker for pancreatic islet transplantation (Papas 2009). Therefore, investigating the potential use of EPR oximetry for assessing the metabolic integrity of stem cells prior to injection would be beneficial for cellular therapies.

Overall, this part of our study (Danhier 2013a) showed that detached cells undergo important metabolic stresses, leading to significant cell death when cells were kept in suspension for extended periods of time. Such important information was completely ignored in our magnetic cell tracking study. Yet, SPIO-labeled cells delivered in the systemic circulation are most likely submitted to similar stresses and the majority of injected cells most

probably die after injection. As a consequence, indirect strategies (such as BLI) providing information on cell function/viability turn out to be crucial in metastasis studies.

II.7. Need for multimodal and molecular imaging approaches

Our work strengthens the fact that no single imaging modality currently meets all the requirements to be an ideal cell tracking method. PET has limited spatial resolution, optical imaging has a poor imaging depth, and MRI is rather insensitive except under some circumstances such as brain imaging using iron oxides (Heyn 2006). Multimodal imaging aims to combine different imaging methods to overcome intrinsic limitations. For preclinical studies, fluorescence imaging may be used with MRI for combining the advantages of both methods, i.e., the sensitive detection of fluorescent signals with the high spatial resolution of MRI (McCann 2009). In clinical oncology, the development of PET/MRI aims to improve the detection and characterization of malignant lesions (Torigian 2013). Multimodal approaches will also be warranted for cell tracking applications. With regard to direct cell tracking methods, the use of iron oxides grafted with PET and NIR probes would be useful for discriminating *in vivo* labeled cells from endogenous confounding factors (Xie 2010). Concerning indirect strategies, fusion reporter strategies, i.e., two or more reporter genes linked with short sequences and driven by a single promoter, allow the simultaneous long-term monitoring of cells using PET, fluorescence imaging and BLI (Ray 2007).

Although improving cancer diagnosis was not the aim of this thesis, MR scans of the mouse brains performed several days after the intracardiac injection of 4T1 cells raise questions (Danhier 2013c). Brain metastases affect 15 to 40 % of cancer patients and conventional MRI is one of the most sensitive clinical diagnostic procedure used routinely for assessing their presence (Fink 2013). In this work, T₂-weighted images performed at ultra high field compared to clinical scanners (11.7 T *versus* 1.5-3 T) with an almost microscopic resolution (~60 µm *versus* 1 mm for clinical MRI) failed to reveal the presence of micrometastases. These results illustrate the issues associated with the minimal residual disease, i.e., a cancer patient may be clinically healthy but host several micrometastases acting as time bombs. This situation justifies the efforts made to improve the detection thresholds of imaging techniques. MRI cell tracking and molecular imaging are related techniques. MRI cancer cell tracking is obviously not intended for diagnosis, whereas on the other hand, molecular imaging is well adapted to solve these issues. For instance, MR molecular imaging using iron oxides targeting the adhesion molecule VCAM-1 was

demonstrated to detect brain metastasis volumes three order of magnitude smaller than the volumes detected using conventional MRI (Serres 2012).

In conclusion, the field of applications of MRI cancer cell tracking is restricted to the assessments of the arrest of CTCs at sites distant from a primary tumor, and the study of the dormancy phenomenon. On the other hand, the ability to image and quantify isolated cells, even *ex vivo*, will be key for the development of cell-based therapies. The important translational potential of MRI cell tracking for cell-based therapies is in fact one of the main advantages of this approach over other imaging modalities. In this regard, magnetic cell tracking approach (EPR and MRI) may constitute a straightforward and robust method for assessing the delivery of stem cells in preclinical models.

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