Control of ligand-binding specificity using photocleavable linkers in AFM force spectroscopy

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ABSTRACT

In recent decades, atomic force microscopy (AFM) and in particular the force spectroscopy mode has become a method of choice to study biomolecular interactions at the single-molecule level. However, grafting procedures as well as determining binding specificity remain challenging. We report here an innovative approach based on a photocleavable group that enables the *in situ* release of the ligands bounded to the AFM tip and thus allows direct assessment of the binding specificity. Applicable to a wide variety of molecules, the strategy presented here provides new opportunities for studying specific interactions and for delivering single molecules with high spatiotemporal resolution in a wide range of applications including AFM-based cell biology.

Over the last decades, AFM has proved to be one of the main techniques for the nanoscale characterization and manipulation of biological specimens in physiological conditions thanks to the combination of its high spatiotemporal resolution and its ability to work in controlled atmospheres[1]. Rapidly, force spectroscopy mode developments together with the ability to functionalize the AFM tip with biological moieties covalently attached opened new avenues to probe the forces involved in bio-interactions down to the piconewton (pN) range and to extract the association dissociation kinetics through established biophysical models[2]. In addition, technological advances in force-distance curve-based AFM (FD-based AFM) enable us to correlate the biomolecular force characterization to simultaneous high-resolution images of biological structures in native conditions[3]. As a result, AFM became one of the most versatile tools for ligand-receptor single-molecule force spectroscopy, which can be performed on systems ranging from biomolecule-grafted model surfaces, to cellular receptors, bacterial and mammalian cells[4]. Moreover, nowadays fluorescence microscopy techniques are routinely implemented in a variety of commercial AFM setups, allowing structural identification of the biological targets by cell biology approaches and further expanding the range of applications of the technique[5].

Notwithstanding the huge progress AFM has witnessed over the years, some of the most fundamental limitations of AFM-based force spectroscopy have not yet been overcome. Mainly, the technique still suffers from uncertainties linked to the identification of the specific interactions, established between the ligand and its specific receptor, among all the inevitable non-specific interactions[6,7]. The latter are generated from interactions of the tip and/or of the immobilized ligand with surface components other than the receptor of interest (*e.g.* spacers, bare surface areas for model substrates and other biomolecules in the case of more complex systems). The proportion of non-specific interactions depends on the complexity of the investigated biological systems, *i.e.*

from receptor-grafted surfaces to lipid membrane models and cells, in which having multiple spurious interactions becomes the rule rather than the exception. Current strategies to differentiate specific and unspecific interactions rely on control experiments using either non-functionalized AFM tips and/or blocking experiments in which free ligands in excess are injected to prevent the specific interaction (ligand itself, antagonist or antibody)[8, 9]. Nevertheless, these strategies do not always lead to convincing results, are usually time and cost-consuming.

Here, we present an optochemical approach taking advantage of the combination of AFM and fluorescence microscopy to control the release of biomolecules from the AFM tips *via* a photocleavable group inserted into the polyethylene glycol (PEG) spacer during the tip functionalization (see Scheme 1). The insertion of a photoresponsive *o*-nitrobenzyl unit[10, 11] within the hydrophilic PEG spacer enables us to achieve post-measurement a light-induced dissociation of the covalently attached ligand. Similar linker cleaving strategies have been proposed before, but they either require the addition of reducing agents potentially unsuitable for complex biological systems[12] or are limited to gold-coated AFM cantilevers, which restrict the range of applications[13]. In our experimental setup, the light source is a standard mercury-arc lamp equipped with a G 365 band pass excitation filter, routinely used for fluorescence dyes in the ultraviolet (UV) range such as the 4',6-diamidino-2-phénylindole (DAPI) cell nuclear stain, and therefore widely available on most commercial microscopes. This easy-to-implement strategy provides a practical way to introduce internal controls in AFM-based force spectroscopy experiments and opens new avenues to bio AFM-based applications.

As a first *proof-of-concept* of our approach, we investigated the well-characterized avidin-biotin interaction[14]. We functionalized the AFM tips with biotin groups using the heterobifunctional PEG linker with the photocleavable group using a multistep procedure (see Figure S1 in the Supporting

Information) and probed the interactions with avidin molecules physisorbed on glass (Figure 1a-d). In parallel, biotin-functionalized AFM tips lacking the photocleavable group were used as a negative control (Figure 1e-h). For both photocleavable (Figure 1b-c) and control tips (Figure 1e-f), rupture forces in the range of 50-100 pN were detected, in good agreement with values reported in the literature for avidin-biotin interactions[14] (examples of FD curves are presented in Figure S2 in the Supporting Information). Remarkably, upon 5 min irradiation of the photocleavable AFM tips at 365 nm *in situ*, the frequency of rupture forces in this range (50-100 pN) was drastically reduced (Figure 1d), while no decrease was observed for the binding probability of the biotin-functionalized control tips (Figure 1h). These results clearly indicate an efficient photolysis of the photoreactive linker leading to a controlled switch-off of the biotin-avidin interactions *in situ*. Notably, direct irradiation of the functionalized AFM tips through the glass substrate does not lead to significant sample damage, as evidenced by the control experiment for which the binding probability was not altered after light exposure. A higher binding probability is observed with the photocleavable tips in comparison with the control tips before photocleavage (Figure 1d and 1h). This suggests that the linker structural change imparted by the insertion of the photocleavable spacer could allow more orientation freedom for biotin, resulting in a higher number of detected avidin-biotin interactions.

Next, similar experiments were performed on a His_6-peptide – *tris*-NTA model system (Figure 2). In this setup, photocleavable (Figure 2a-d) and control (Figure 2e-h) *tris*-NTA-functionalized AFM tips were used to probe the interaction with His_6-peptide-functionalized surfaces (see Figure S3 in the Supporting Information). As for avidin-biotin, after an initial assessment of *tris*-NTA–His_6 rupture forces and binding probability, the functionalized AFM tips were exposed to UV light *in situ* for 5 min to release the *tris*-NTA groups. *Tris*-NTA was found to bind the His_6-peptide with

rupture forces in the 50-200 pN range[15, 16] and binding probabilities up to ~80%, indicating a high peptide surface coverage in our experimental conditions. Similarly to what we observed for the avidin-biotin system, the binding probability of the *tris*-NTA-functionalized photocleavable tips decreased considerably after UV light exposure, while no variation was found for the *tris*-NTA control tips, confirming that biomolecular interactions can be efficiently suppressed *in situ* through controlled photocleavage of the ligand from the AFM tips.

Finally, to demonstrate that our strategy is applicable to more complex biological systems, we tested the feasibility of the photocleavable linker to study single-virus binding to living mammalian cells. To this end, we functionalized AFM tips with fluorescently labeled reoviruses, whether or not using a photocleavable group (see Figure S4 in the Supporting Information). The functionalized tips were used to probe virus interactions with Chinese hamster ovary (CHO) cells overexpressing the junction adhesion molecule A (JAM-A), which we recently showed to be an efficient binding receptor for reoviruses[17]. Using FD-based AFM combined with laser scanning confocal microscopy (Figure 3), the AFM tip was raster scanned on the cell surface and rupture forces were extracted for each pixel. Imaging of the AFM tip using laser-scanning confocal microscopy showed that after 5 min of UV light-exposure, reoviruses were efficiently removed from the AFM tips bearing the photocleavable groups while remaining attached for AFM tips having no photocleavable group (either directly irradiated (Figure 3a) or irradiated through the cell sample (Figure 3b, upper row)). Furthermore, the comparison between AFM topography (Figure 3b, middle row) and adhesion images (Figure 3b, bottom row) revealed that virus release is accompanied by a significant decrease of binding event probability, from ≈12% to ≈3-4%. As a control, consecutive mapping with AFM tips lacking the photocleavable groups did not show this behavior, with a stable binding event probability around ≈10%, confirming that the virus remains

attached for several consecutive maps. Indeed, although a slight decrease of the fluorescence intensity is observed, likely due to photobleaching, control tips did not seem to significantly release reoviruses (Figure 3c and 3d, upper row), nor loosing cell-binding capability after light-exposure (Figure 3d, middle and bottom rows), suggesting that both phenomena are specifically related to the linker cleavage and virus release. These *proof-of-concept* experiments presented here confirm the versatility of the photocleavable approach. In addition, preliminary results demonstrated the possibility to release virions at a defined location on a cell layer upon photocleavage (Figure S5), opening new opportunities to further AFM- and fluorescence microscopy-based cell-biology applications.

In summary, we presented a practical and versatile optochemical approach to control the release of biomolecules from AFM cantilevers. The method has been applied successfully to both receptors immobilized on model surfaces and to more complex biological systems. The ligand-release upon UV-light exposure appears as a powerful internal control for force spectroscopy experiments. Furthermore, more sophisticated biology-oriented applications for combined AFM-optical microscopy instruments, such as the possibility of releasing ligands at specific cellular locations while following the evolution of cellular responses is envisaged in the near future.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge. Additional details on the experimental methods, chemical characterization of the Fmoc-photo-NHS group and figures showing the coupling steps from a chemical point of view (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AFM, atomic force microscopy; pN, piconewton; FD, force-distance; UV, ultraviolet; DAPI, diamidino-2-phénylindole; PEG, polyethylene glycol; CHO, Chinese hamster ovary; JAM-A, junction adhesion molecule A

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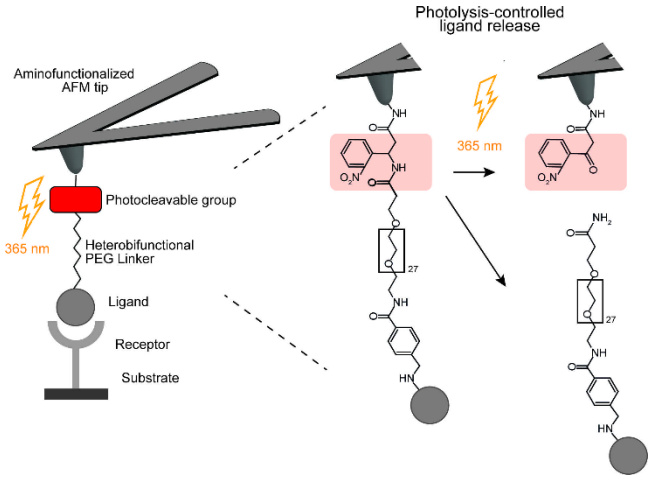
FIGURES



Scheme 1. Schematic and experimental concept of an AFM tip functionalized with a photocleavable group, followed by heterobifunctional PEG linker attachment and ligand coupling to probe the interaction with a cognate receptor before and after controlled ligand release via photolysis at 365 nm.

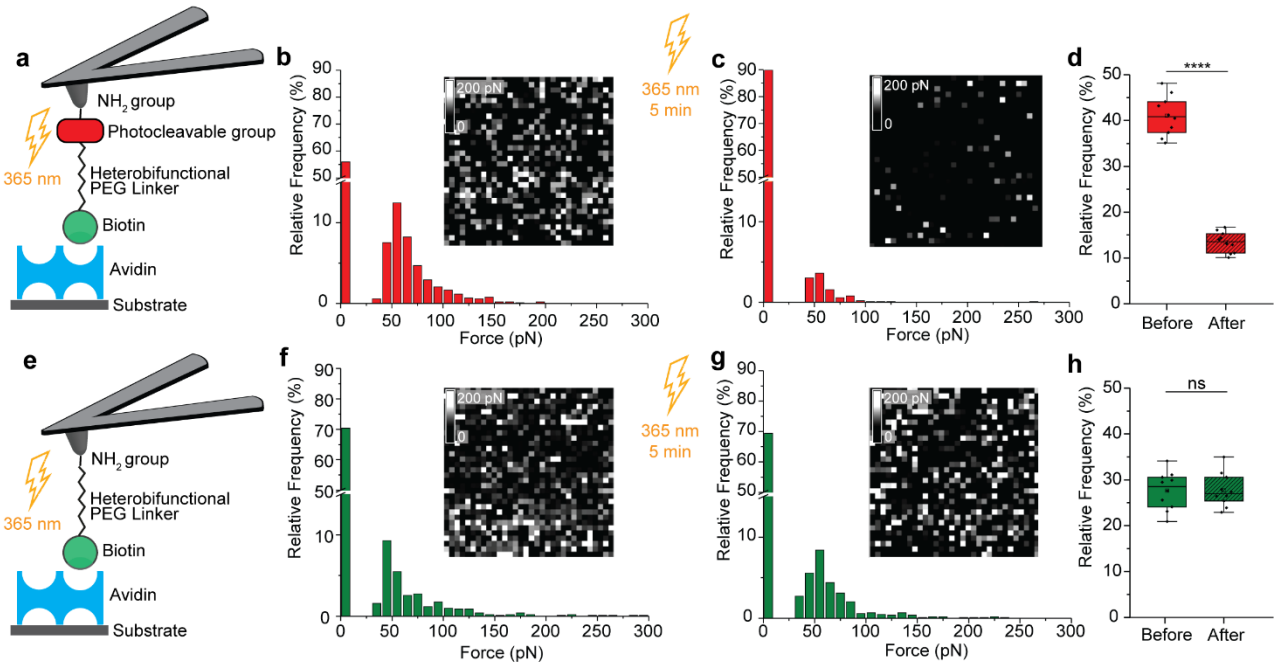


Figure 1. Force spectroscopy of single avidin – biotin interactions acquired with photocleavable (a-d) and control (e-h) AFM tips. (a) Scheme of the photocleavable tips system. (b, c) Adhesion force histogram and map (insert; 5 μm x 5 μm) of the avidin – biotin interaction before (b) and after (c) photolysis at 365 nm for 5 min. (d) Box plot of the relative binding frequency before and after (dashed) photolysis showing a significant decrease in binding. (e) Scheme of the control tips system. (f, g) Adhesion force histogram and map (insert; 5 μm x 5 μm) of the avidin – biotin interaction before (f) and after (g) photolysis at 365 nm for 5 min. (h) Box plot of the relative binding frequency before and after (dashed) photolysis showing no significant decrease in binding. Data are representative of at least n = 3 independent experiments, with a minimum of n = 10 analyzed adhesion maps before and after photolysis. ns, $P > 0.05$; ****$P < 0.0001$; determined by two-sample t-test in Origin.

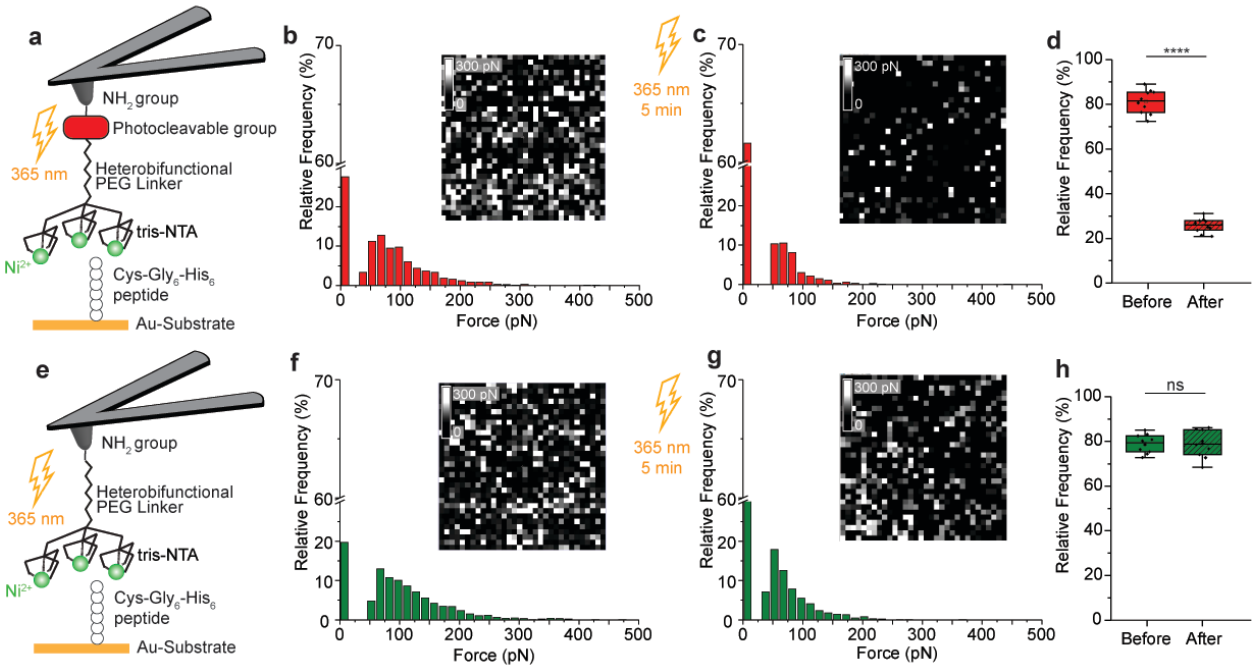
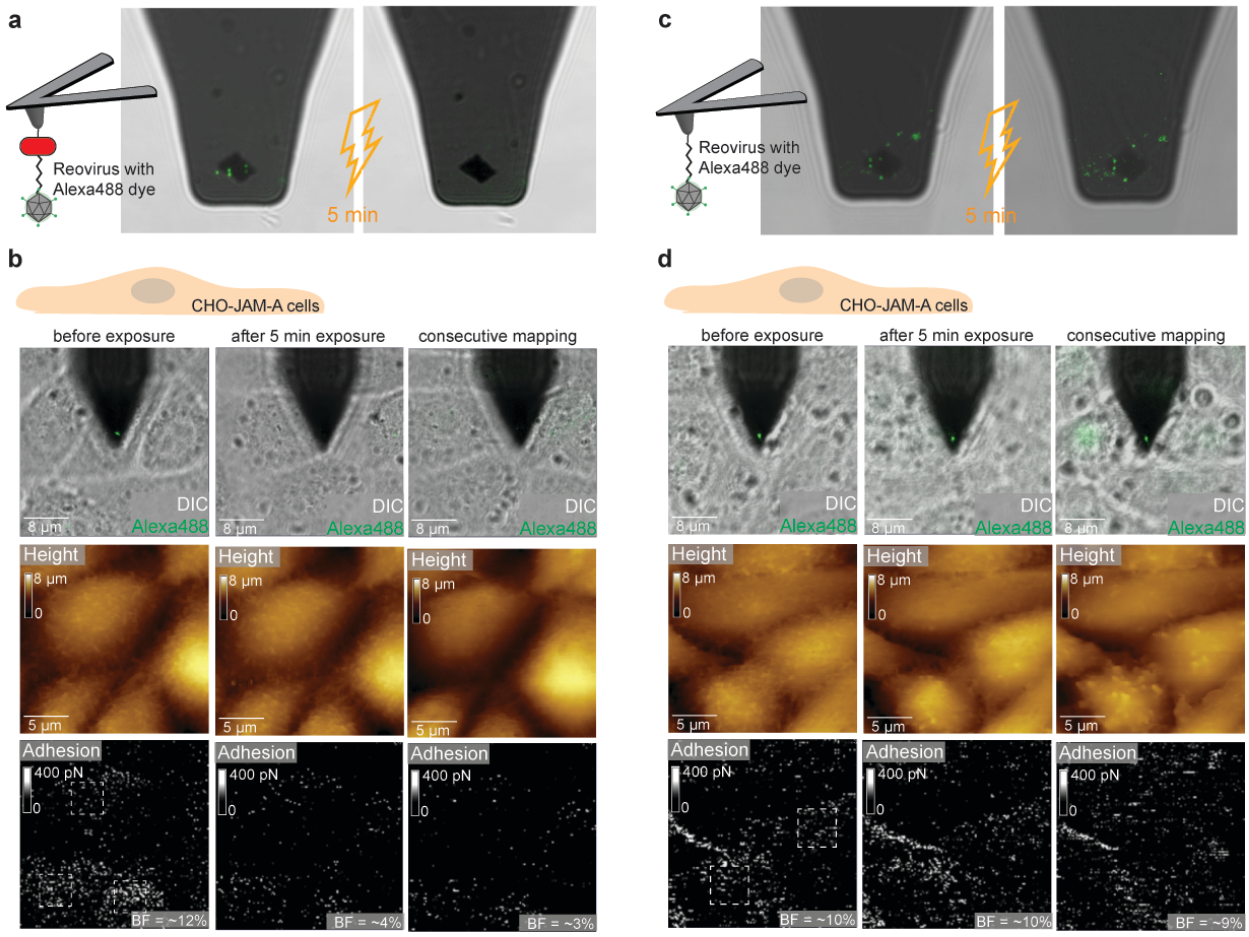


Figure 2. Force spectroscopy of single His6-peptide – *tris*-NTA interactions acquired with photocleavable (a-d) and control (e-h) AFM tips. (a) Scheme of the photocleavable tips system. (b, c) Adhesion force histogram and map (insert; 5 μm x 5 μm) of the His_6-peptide – *tris*-NTA interaction before (b) and after (c) photolysis at 365 nm for 5 min. (d) Box plot of the relative binding frequency before and after (dashed) photolysis showing a significant decrease in binding. (e-j) Control experiment without photocleavable group present. (e) Scheme of the control tips system. (f, g) Adhesion force histogram and map (insert; 5 μm x 5 μm) of His6-peptide – *tris*-NTA interaction before (f) and after (g) photolysis at 365 nm for 5 min. (h) Box plot of the relative binding frequency before and after (dashed) photolysis showing no significant decrease in binding. Data are representative of at least n = 3 independent experiments, with a minimum of n = 10 analysed adhesion maps before and after photolysis. ns, $P > 0.05$; ****$P < 0.0001$; determined by two-sample t-test in Origin.



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249 **Figure 3.** Proof of concept (a-b) and control (no photocleavable group, c-d) experiments on
250 largescale molecules (reovirus) interacting with specific reovirus attachment receptors (sialic acid,
251 JAM-A) probed on living CHO-JAM-A cells (see Supporting Information). (a) AFM tip
252 functionalized with a fluorescently labelled (Alexa488, green) reovirus releases most of the single
253 viruses after photocleavage and shows a significant decrease in binding frequency after probing
254 their interaction on JAM-A overexpressed CHO-cells (highlighted areas in b). (c) The control
255 experiment shows almost no virus release after photocleavage as well as no significant drop in
256 binding frequency (highlighted areas in d).

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