universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Towards the Mechanism of Protein-Peptide Photounbinding

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Klaus Neumüller
Matrikel-Nummer:	0305217
Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie / Genetik - Mikrobiologie
Betreuerin / Betreuer:	Dr. Katrin Heinze

Wien, im Juni 2009

Directory

Towards the Mechanism of Protein-Peptide Photounbinding

1. Introduction **1**
1.1. State of the art 1
1.2. How to probe photounbinding in solution 3
1.3. Calmodulin – Calcium/Calmodulin-Dependent Protein Kinase II system 5
1.4. Photobleaching 8
1.5. Fluorescent dyes 9

2. Material and Methods **11**
2.1. Reagents 11
2.2. Cell culture 12
2.3. Calmodulin and Calmodulin dependent kinase (CKII) peptides 13
2.4. Staining protocols 14
2.5. Immobilization strategy 16
2.6. Photounbinding assays 17
a. CaM/CKII 17
b. phalloidin/actin 17
2.7. Data acquisition 18
2.8. Computer based data analysis 18

3. Results **21**
3.1. Fluorescently labeled Calmodulin undergoes unbinding from CKII peptides 21
3.2. Photounbinding of labeled CaM from CKII peptides is dependent on the dissociation constant 24
3.2.1. Mathematical correction of CaM-A647 rebinding values 29
3.3. Photounbinding of labeled Calmodulin by two-photon excitation 31
3.4. Photounbinding requires an absorbing or fluorescent label 33
3.5. Photounbinding is directly linked to photobleaching 34
3.6. Photounbinding of CaM is not limited to a specific absorption wavelength or fluorophore 38
3.7. Key-controls and calibration measurements 39
3.7.1. Photounbinding does not occur for covalent bonds 39
3.7.2. Photounbinding does not occur with GFP-actin fusion proteins 41
3.7.3. Rebinding of identically labeled Calmodulin 42
3.7.4. Determination of fluorescence quenching by label interactions of A488 and A647 43
3.7.5. Determination of unspecific binding of Calmodulin to the CKII peptide coated glass surface 45
3.8. Photounbinding of fluorescent phalloidin in cells 48

4. Discussion and Outlook **57**

5. References **62**

6. Abbreviations **65**

Summary **67**

Zusammenfassung **68**

Acknowledgements **70**

Curriculum Vitae **71**

1. Introduction

1.1. State of the art

Fluorescent dyes or genetically encoded fluorescent probes are commonly used in biological experiments. Although fluorescent tags are essential for a broad range of experimental studies, it is important to keep in mind that properties of proteins or nucleic acids can be influenced by fluorescent conjugates. The chemical and physical properties of the conjugated dye can for example affect the structure and motility of proteins or nucleic acids, yet it is often unclear to which extent light excitation of the fluorescently tagged specimen biases the results of fluorescence imaging and other optical studies.

Phototoxic reactions have been demonstrated to bias measurements and are major limitations in live cell fluorescence microscopy (Hoebe et al. 2008), especially for light excitation beyond the fluorescence saturation limit of a fluorophore. For techniques such as FRAP or FLIP[1] that use photobleaching as a tool , it has been shown that these phototoxic effects can be exerted not only on the illuminated cell but also on neighboring fluorescent cells (Dobrucki et al. 2007).

Photophysical and photochemical effects such as the production of reactive oxygen species (ROS) by fluorescent proteins under illumination, can cause problems ranging

[1] FRAP allows to measure diffusion events within cells. The proteins or molecules of interests are labeled with a fluorescent dye. A short laser pulse is used the photo-bleach a certain region within a cell for example, whereas it is assumed that the fluorescent dyes are converted into a non-fluorescent state. The bleached area gets "refilled" with other fluorescently labeled proteins or molecules and the increasing fluorescence in this area is measured. Diffusion rates can be calculated based on the restored fluorescence.

FLIP is a technique that enables measurements of movement or diffusion of fluorescent molecules inside a cell or along a cell membrane. A certain region of a cell membrane can be bleached (bleaching is done continuously) by laser excitation for example. The motion of labeled molecules into or along the membrane restores the fluorescence in the bleached region whereas the fluorescence around the bleached region decreases due to exchange of bleached and non-bleached molecules. This technique allows evaluating directions of intracellular trafficking.

For details on how these techniques can be biased by light excitation see *Results* and *Discussion*.

from artifacts in dynamic measurements to cell death (Remington J.S. 2006). However, these effects can also find useful applications. For example, KillerRed (a genetically encoded photosensitizer) can, due to its high levels of ROS production (Bulina et al. 2006), be used to selectively destroy cells and to inactivate specific proteins (CALI) upon irradiation with green light.

Further insights in photochemical and photophysical effects may lead to the development of other new useful tools with a broad range of applications such as real time biosensors (Remington J.S. 2006).

Recent studies have shown that labeled binding partners can be dissociated by light excitation. In these studies it was observed that labeled antibody-antigen (Heinze et al. 2009) and toxin-target (Akaaboune et al. 2002) interactions can be unbound by light.

The basic mechanisms of this phenomenon, referred to as photounbinding, is not yet fully understood. Detailed knowledge about the process and mechanism of photounbinding would not only allow a systematic improvement of quantitative fluorescent studies (e.g. FRAP, FLIP), but also open the door to using the photounbinding phenomenon as a tool in biomedicine (*see Discussion*).

In the presented study we characterize photounbinding in solution and in cells for two molecular systems. These were Calmodulin-peptide binding using peptides with various binding affinities, and intracellular photounbinding in fixed cells where we studied the dissociation of fluorescently tagged phallotoxins from actin filaments.

Photounbinding was characterized after one and two photon excitation. Within a small volume the impact of laser illumination is particularly relevant for 2PE given that the required laser power for two photon excitation is approximately one order of magnitude higher than for one photon excitation (Kim et al. 2007) and that 2PE shows a square dependence on the excitation power (which narrows the power range between fluorescence saturation and bleaching significantly). The high photon flux used for 2PE can also potentially lead to higher-order photobleaching (Chen et al. 2002), which makes bleaching within the focal volume more problematic (Heinze et al. 2009).

It is important to note that photounbinding could be misinterpreted as photobleaching since these effects are hard to distinguish between in a typical imaging setup. Thus, light induced unbinding may remain “masked” and be misinterpreted as photobleaching in some quantitative fluorescence studies, especially when using photobleaching as a tool (e.g. FRAP or FLIP). Therefore, part of the presented study involved the development of

a binding assay suitable to distinguish the loss of a binding partner (photounbinding) from the loss of fluorescence by photobleaching.

1.2. How to probe photounbinding in solution

In this work several studies were performed to analyze light induced unbinding of fluorescently labeled Calmodulin (CaM) from various immobilized CaM binding peptides. The idea behind this is that light excitation can induce the dissociation of a labeled binding partner (e.g. CaM-A488) from its ligand (e.g. CKII-peptides). Here, peptide-immobilization on a coverglass allows one- and two photon excitation in defined regions with a Laser Scanning Microscope of the protein peptide (CaM-A488/CKII-peptide) layer. Bleached CaM-A488 would remain bound to the peptide coated surface whereas light induced dissociation would result in a vacant binding site, which could be visualized by subsequent rebinding of a differently labeled CaM (fig.1.a). With this in mind, an assay with a CaM/CKII-peptide binding system was designed for this study (fig.1.b).

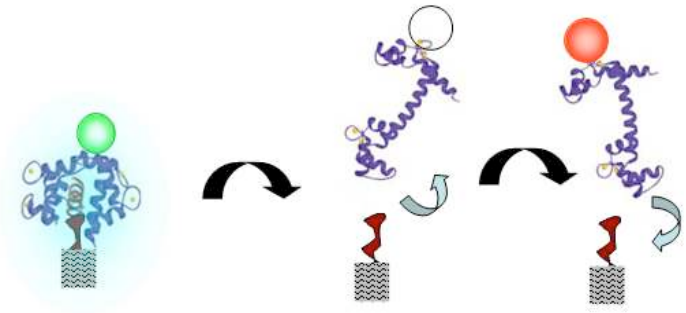


Fig. 1.a: scheme of CaM-A488 – photounbinding from an immobilized ligand (CKII-peptide); following light excitation labeled CaM can be dissociated which results in a vacant binding site; photounbinding can be visualized by subsequent rebinding of differently labeled CaM.

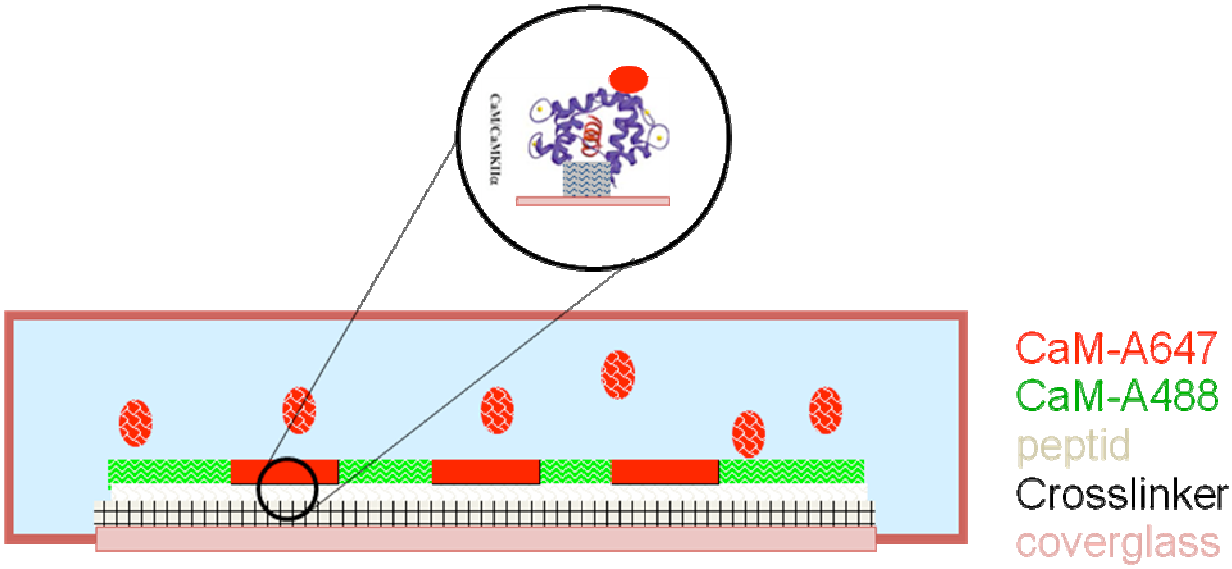


Fig. 1.b: scheme of photounbinding assay used for this study; CKII peptides were attached to a glass surface via an SM(PEG)8 crosslinker; CaM-A488 was incubated on the CKII-peptide surface and dissociated by laser excitation; following laser illumination, the surface was incubated with CaM-A647 to visualize free binding sites in the illuminated regions.

The CaM/CKII peptide system used (see below) allows measurements concerning the dependence of photounbinding on the dissociation constant within a particular molecular system. This is possible because the CaM/CKII peptide – complexes have different dissociation constants depending on the length of the CKII peptide.
By performing the photounbinding measurements (assay *see above*) with a confocal microscope allowed for the determination of the amounts of bleached/photo-unbound and rebound CaM.

In this work emphasis was put on the characterization of the molecular mechanisms behind photounbinding. Studies were done in several binding systems (CaM/CKII, phalloidin/actin and transgenic GFP actin filaments) with different labels and excitation wavelength which show that photounbinding is a universal phenomenon, not restricted to a specific interaction or excitation wavelength. Studies based on different binding systems allowed further characterization of fundamental properties of light induced dissociation. Further insights into basic mechanisms will help to understand the overall process of photounbinding. In this work emphasis was put on:

- the characterization of the dependence of photounbinding on the dissociation constant
- studies concerning the link of unbinding to photobleaching
- studies concerning the label-dependence of photounbinding
- the characterization on photounbinding within cells (phalloidin/actin interaction)
- studies concerning the dependence of photounbinding on the nature of the chemical bond between binding partners (e.g. covalent versus non-covalent)

The design and development of proper assays (see above) which allow quantification of photounbinding enabled us to further characterize these molecular mechanisms.

1.3. Calmodulin – Calcium/Calmodulin-Dependent Protein Kinase II system

To analyze the dependence of photounbinding on the dissociation constant we chose the CaM/CKII-peptide system. This is an ideal system for such studies because the dissociation constant depends on the length of the CKII peptide. Different CKII peptides (see *Material and Methods*) were synthesized for this study, which allowed measurements of the dependence on the dissociation constant within this particular molecular system.
CaM is a 17 kDa, highly conserved protein. The protein has an N- and C-terminal lobe connected through a flexible linker. Each lobe contains a pair of EF-hand motifs (a helix-loop-helix structural domain), allowing it to bind four Ca^{2+} ions in total (Swulius et al. 2008). When loaded with Calcium, it can bind to various targets, amongst them, the Calcium/Calmodulin-Dependent Protein Kinase II. Fig. 2 shows a model of a CaM/CKII peptide interaction.

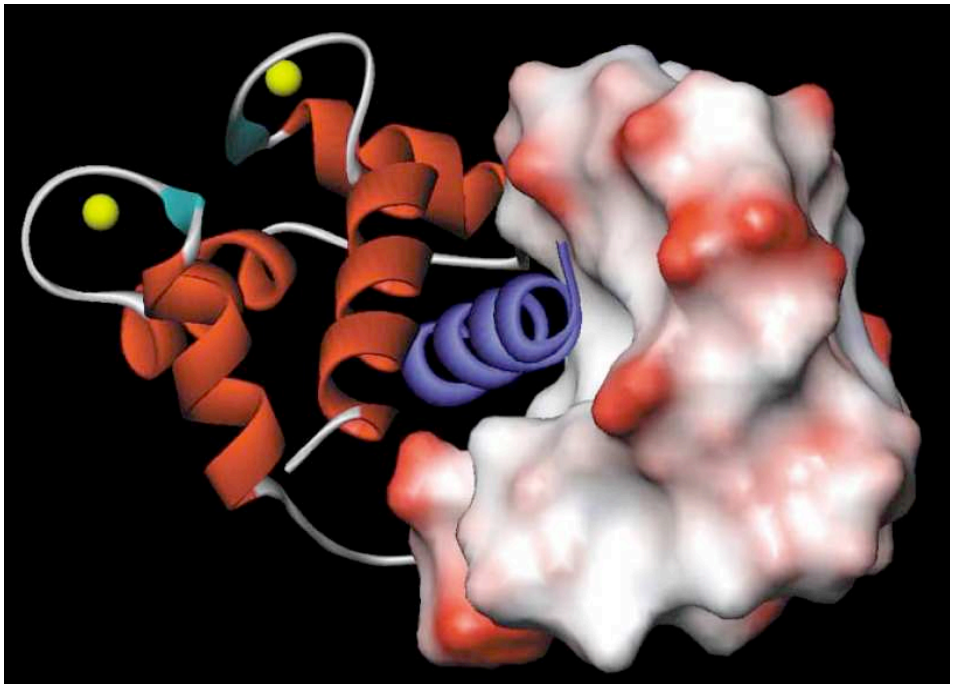


Fig. 2: model of the CaM/CKII peptide interaction; the N-terminal lobe of CaM is rendered as a ribbon, the C-terminal lobe is represented by its electrostatic surface, the CKII peptide is shown as a blue ribbon (Vetter and Leclerc 2003).

The CKII peptide is bound in a α-helical conformation. It is engulfed by CaM into a hydrophobic channel. The CaM/CKII interaction is largely driven by hydrophobic interactions between hydrophobic anchor residues of the peptide with the hydrophobic surface cavities of CaM (Vetter and Leclerc 2003).

CaM-kinases are critical for a widespread range of cellular functions and they can serve as inducer or modulator of many cellular responses to Ca^{2+} signals (Hudmon and Schulmann 2002). They influence cellular processes as for example Apoptosis, learning, memory formation, reorganization of the cytoskeleton or gene transcription.

Calcium/Calmodulin-Dependent Protein Kinases II subunits form stable dodecamers (fig. 3). The activity of each subunit can differ depending on the functional state. Intrinsic auto-inhibition domains can keep the Calcium/Calmodulin-Dependent Kinase inactive in its basal state. An active state can be induced by the binding of Ca^{2+}/CaM. This leads to auto-phosphorylation of Thr^{286} (fig. 4), which results in a new state, in which CaM becomes trapped. This allows the enzyme to stay active for several seconds even though Ca^{2+} levels have dropped (Kim et al. 2005; Meyer et al. 1992)

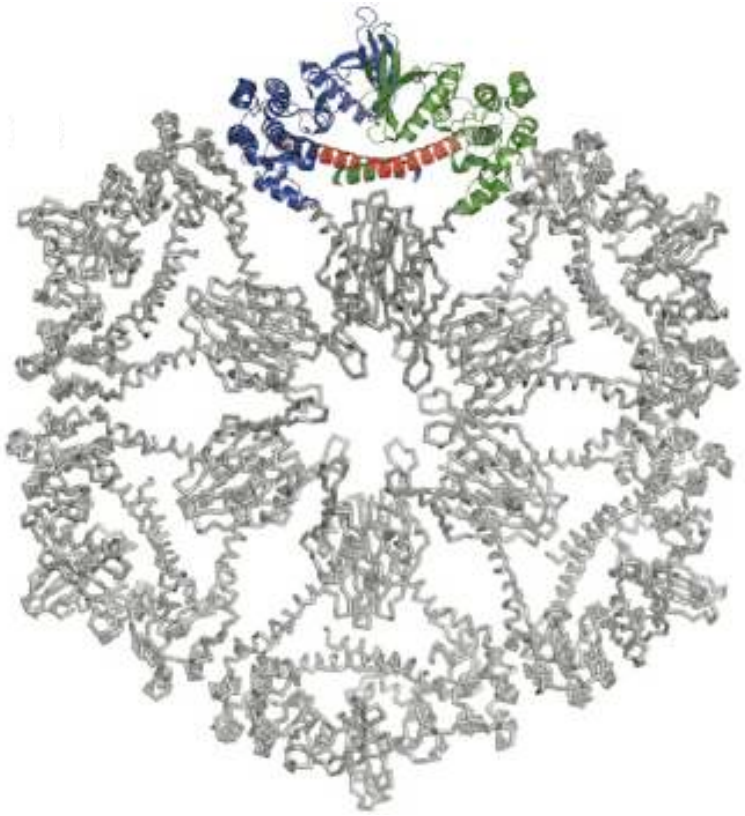


Fig. 3: Reconstruction of the CK-II holoenzyme (Pellicena and Kuriyan 2006)

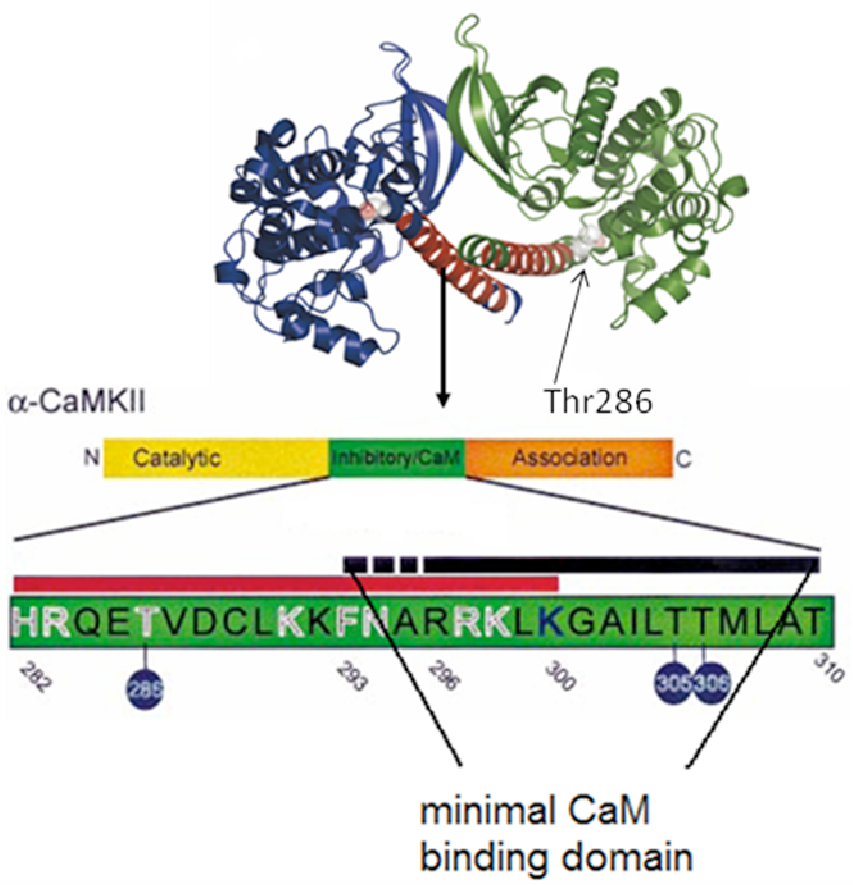


Fig. 4: CKII dimer, the major regulatory autophosphorylation site, Thr 286 is shown for one of the monomers; the minimal CaM-binding domain is shown in black (Pellicena and Kuriyan 2006)

Synthetic peptides can kinetically mimic low affinity (unphosphorylated) and high affinity (phosphorylated) binding of CaM-kinase II to CaM (Waxham et al. 1998). Peptides which resemble high and low affinity states were synthesized and used for photounbinding measurements in this study (fig. 5).

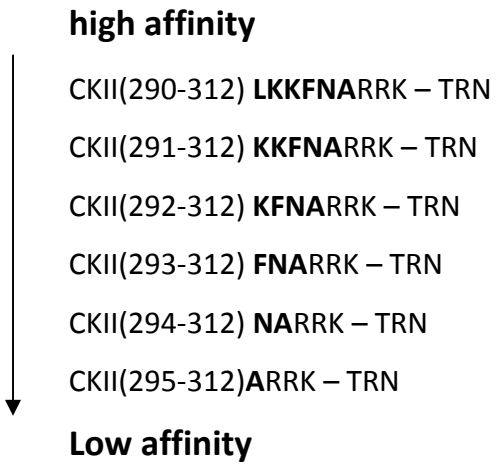


Fig. 5: CKII peptides kinetically mimic high and low affinity binding states of CKII to CaM, depending on their length

1.4. Photobleaching

As already mentioned, it is difficult to distinguish photounbinding from bleaching without an adequate assay. Therefore light induced unbinding can easily be misinterpreted. In this work it was observed that photounbinding and photobleaching occur together at all tested laser intensities (see *Results*). On account of these we analyzed if bleaching and unbinding are linked processes (see *Results 3.5.*). The process of photobleaching has been extensively studied (White et al. 1999). Here we only provide a brief summary of details relevant to our studies.

The photochemical destruction of a fluorescent dye is referred to as photobleaching. It is a dynamic process in which fluorophores are destroyed through the excitation light and lose their fluorescence (Song et al. 1995).

Photobleaching is dependent on several factors like the intensity and the energy of the excitation wavelength or the molecular structure of the fluorophore. Additionally the

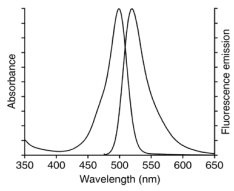
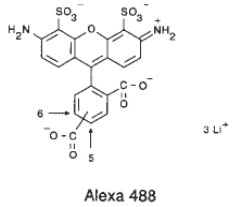
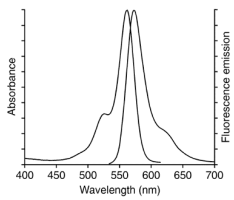
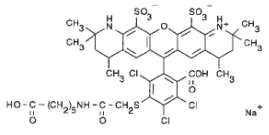
chemical surroundings can influence photobleaching as well. Ascorbic acid for example can reduce bleaching of fluorescent dyes and photounbinding (see above) for 2PE (Dittrich et al. 2001).

There are fluorescent dyes which are more photo-stable than others.

1.5. Fluorescent dyes

Several fluorescent dyes were used in this study. The Alexa dyes are quite stable dye molecules and were used to investigate light induced unbinding of labeled CaM and phalloidin.

Table 1 shows the chemical structures and spectra of the fluorescent dyes that were used in this work:

fluorescent dye	molecular weight	em. max. (nm)	abs. max. (nm)	Spectra	chemical structure
Alexa Fluor 488	643,41	517	494		 Alexa 488
Alexa Fluor 546	1079,39	570	554	Alexa Fluor 546 goat anti–mouse IgG in pH 7,2 Puffer 	 Alexa 546

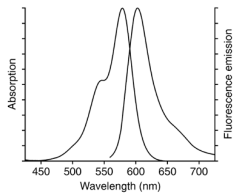
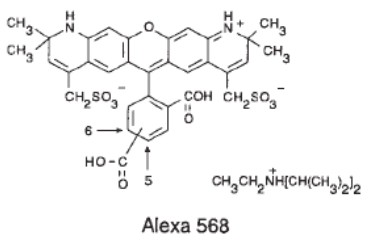
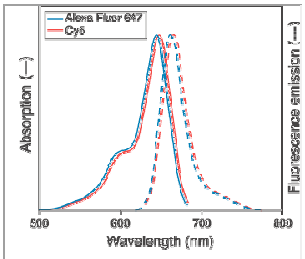
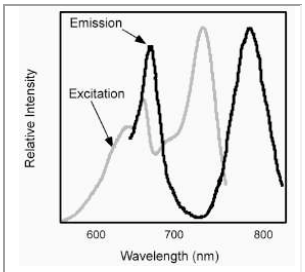
Alexa Fluor 568	792	603	578		
Alexa Fluor 647	1200	647	632		n.a.
APC-Cy7	~85 kDa	650	767		n.a.

Table 1: chemical structures and spectra of dyes that were used in this work (Panchuk-Voloshina et al. 1999; www.invitrogen.com)

2. Material and Methods

2.1. Reagents

buffers PBS (phosphate-buffered saline)
coupling buffer (1x PBS, EDTA 5mM)
CaM buffer (25mM MOPS, 150 mM KCl, 0.5 mM $CaCl_2$, 0.1 mg/ml BSA pH ~ 7.2)
blocking buffer (25 mM MOPS, 2% BSA)

chemicals crosslinker: $SM(PEG)_8$ (Thermo Scientific; product number 22108)

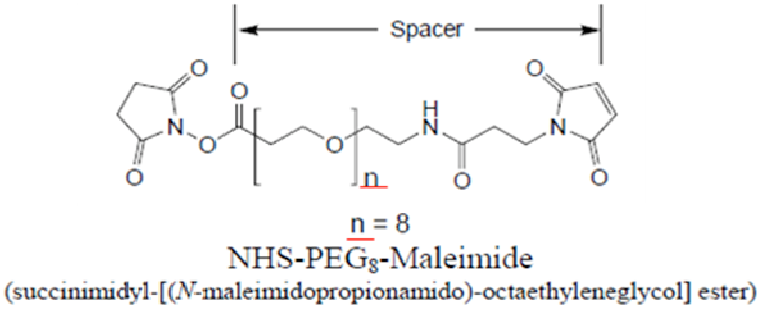


Fig. 6: SM(PEG)8 crosslinker (www.piercenet.com)

3-Aminopropyltriethoxysilane (Thermo Scientific; product number 80370)

dry Acetone (max. 0.01 % H_2O; Secco Solv)
Nitric acid 60 %, silica, ascorbic acid, Triton X-100, PFA (Paraformaldehyde)

Antibodies, Streptavidin

Goat anti-GFP – biotinylated (abcam, ab6673-100)
Streptavidin APC-Cy7 (Prod. Nr. 554063)
Calmodulin Antibody (Calmodulin Ab-4; mouse monoclonal antibody; MS-1268-P0)

Anti-mouse IgG – Alexa Fluor 568 (Invitrogen)
Fibroblast [1B10] antibody (GeneTex; Prod. Nr. GTX11333]
Goat anti-mouse IgM Alexa 488 (Invitrogen, Prod. Nr. A-21042)
Goat anti-mouse IgM Alexa 647 (Invitrogen, Prod. Nr. A-21238)
Goat anti-mouse IgM Alexa 546 (Invitrogen, Prod. Nr. A-21045)

Phalloidin
Alexa Fluor 488 Phalloidin (Invitrogen, Prod. Nr. A22287)
Alexa Fluor 647 Phalloidin (Invitrogen, Prod. Nr. A22287)
Phalloidin – unlabeled (Invitrogen Prod. Nr. P3457)

Dyes
Alexa Fluor 488
Alexa Fluor 647
Alexa Fluor 568
Alexa Fluor 546
APC-Cy7

2.2. Cell culture

Growth Medium, Reagents

PBS (autoclaved)
DMEM (10 % FCS, 2 mM L-Glutamine)
Trypsin EDTA

Cell Lines

AAV-HT1080; human fibrosarcoma cells
B-16 actin GFP; mouse melanoma cells

Establishing B-16 actin-GFP mouse melanoma and AAV-HT1080 cultures from frozen cells

Frozen cryovials were thawed within 2 minutes in a 37 °C water bath.

The thawed cell suspension was transferred to a 15ml Falcon tube containing growth medium (10 ml) and the cells were collected by centrifugation at 200 x *g* for 3 minutes (RT).

The cells were resuspended in 5 ml growth medium and transferred to a 75-cm^2 tissue flask containing 10 ml growth medium. The flasks were incubated at 37°C and 5% CO_2.

Passaging of Cells

DMEM growth medium, trypsin-EDTA and PBS were prewarmed in a 37°C water bath.

The growth medium was removed of the 75-cm^2 tissue flasks and the cells were washed with 10 ml PBS.

Trypsinization was performed for 2 minutes (5 ml trypsin-EDTA) to release adherent cells.

After two minutes, 5 ml DMEM growth medium was added to inactivate the trypsin.

20 ml of DMEM growth medium were added to a 75-cm^2 tissue flask and 1.5 ml of the cell suspension were transferred to the flask.

The flasks were incubated at 37°C and 5% CO_2.

The cell density was monitored and maintained at 50 % confluence.

2.3. Calmodulin (CaM) and Calmodulin dependent kinase (CKII) peptides

CaM-A488 and CaM-A647 were kindly provided by Prof. M. Neal Waxham (UTMS-H, Houston, TX; USA).

Sequence of rat wildtype CaM:

```
>gi|1334203|emb|CAA32119.1| calmodulin [Rattus norvegicus]
MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFL
TMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYE
```

EFVQMMTAK

The provided CaM carried an Alexa dye at position three, which was altered from Asp to a Cys residue. The change in the amino acid sequence is necessary to attach the Alexa dye to the protein via a disulfide bridge.
CaM was at a concentration of 30.2μM in MOPS (20mM).
To avoid thaw and freezing cycles, CaM was aliquoted and shock frozen in liquid nitrogen.

CKII peptides were synthesized with support of the protein chemistry facility.
The following peptides were synthesized (an N-terminal Cys residue was necessary to immobilize the peptides on the SM(PEG)$_8$ crosslinker):

CKII(290-312)	LKKFNARRKLKGAILTTMLATRNC
CKII(291-312)	KKFNARRKLKGAILTTMLATRNC
CKII(292-312)	KFNARRKLKGAILTTMLATRNC
CKII(293-312)	FNARRKLKGAILTTMLATRNC
CKII(294-312)	NARRKLKGAILTTMLATRNC
CKII(295-312)	ARRKLKGAILTTMLATRNC
CKII(290-312)*	LKKFNARRKLKGAILTTMLATRN

The peptides were purified with HPLC (High Performance Liquid Chromatography) and verified by Mass Spectrometry.

2.4. Staining protocols

Actin staining

AAV-HT1080 cells were cultivated on chambered cover-glasses for 24 hours.

The DMEM growth medium was removed and the cells were washed with prewarmed PBS and fixed in a 4 % PFA PBS solution for 15 minutes at

room temperature. The cells were permeabilized with 0.1 % Triton X-100 for 3 minutes and staining was done according to the protocol on formaldehyde fixed cells by invitrogen (http://probes.invitrogen.com/media/pis/mp00354.pdf) (phalloidin incubation was done for 60 minutes, to ensure saturation of free binding sites).

CaM staining

CaM was bound to a CKII(290-312) coated surface (see *immobilization strategy*).

The Calmodulin coated surface was stained with CaM Ab-4 (IgG), a mouse monoclonal antibody and anti-mouse IgG A568. The CaM-antibody was diluted in PBS to 1μg/ml and incubated on the CaM surface for 30 minutes at room temperature. The surface was rinsed with CaM-buffer and an anti-mouse IgG A568 was incubated on the surface for 30 minutes at room temperature followed by rinsing with CaM-buffer.

GFP staining

Formaldehyde fixed B16 actin-GFP cells (permeabilized with 0.1 % Triton X-100) were stained with anti-GFP-biotinylated/Streptavidin APC-Cy7. The cells were incubated in PBS 1 % BSA for 30 minutes to reduce background staining. Goat anti-GFP (1.1 mg/ml) was diluted in PBS 1%BSA (1:400) and incubated on the cells for 30 min. The cells were washed with PBS for three times and incubated with Streptavidin APC-Cy7 at the same concentration for 30 minutes at room temperature.

Fibroblast surface protein staining on living cells

A fibroblast [1B10] antibody was diluted 1: 500 in PBS (final concentration: 0.4 μg/ml) and incubated on AAV-HT1080 cells for 30 minutes followed by washing with PBS.

Goat anti-mouse IgM Alexa 488 was used as a secondary antibody [dilution:
1:200 in PBS to a final concentration of 5 μg/ml, incubation : 20 minutes (100 μl per 500 ml chamber) followed by rinsing with PBS].
A rebinding pattern could not be seen, due to the observation that most of the cells died during the staining, bleaching and re-staining procedure.

2.5. Immobilization strategy

CKII peptides were immobilized on silylated glass surfaces.

Cleaning of cover-glasses:
Cover-glasses were washed in: ddH_2O (3 x 5 minutes), ethanol (3 x 2-3 minutes), HNO_3 50% (4 hours, on a shaker) and ddH_2O (3 x 5 minutes)
The cover-glasses were tried under a N_2-flow after cleaning.

Amino-silylation/Peptide to glass attachement:
Amino-silylation of cover-glasses was done in a 2 % solution of 3-aminopropyltriethoxysilane in dry acetone. The cover-glasses were immersed in this solution for one minute and rinsed with acetone.
The cover-glasses were assembled in home built glass bottom dishes. Commercially available glass-bottom petri dishes could not be used, because the plastic does not tolerate the required acetone treatment.
The dry cover-glasses were incubated in a DMSO $SM(PEG)_8$ crosslinker (0.5 mM) solution for 30 minutes at RT and rinsed with coupling buffer. Immediately after rinsing, the cover-glasses were incubated with CKII peptides at a concentration of 1mM. The CKII peptides bind to the maleimide group of the $SM(PEG)_8$ crosslinker via an N-terminal Cys residue (incubation was done at 4 °C overnight).

2.6. Photounbinding assays

a. CaM/CKII

This assay allows measurements of unbinding events of labeled CaM from a peptide coated glass surface. Therefore, CKII peptide coated cover-glasses were incubated with CaM-A488 (3μM in CaM-buffer) o.n. at 4 °C to saturate nearly all free binding sites.
Following incubation, the CaM/CKII peptide surface was bleached by one and two photon excitation. A bleaching pattern, with increasing laser intensities was "written" onto to CaM-A488 coated surface. For this purpose laser intensities ranging from a few μW to several mW were applied (see results).
To avoid rebinding of unbound CaM-A488 the glass bottom dishes were filled with CaM-buffer to dramatically dilute unbound CaM.
To visualize free binding sites after photounbinding, the surface was incubated with CaM-A647 for 40 minutes at RT.

b. phalloidin/actin

An AAV-HT1080 human fibrosarcoma cell line was grown on chambered cover-glasses, stained with labeled phalloidin (for details see *staining protocols*) and overlaid with PBS.
Actin filaments were bleached by one and two photon excitation with different laser intensities to induce photounbinding.
Photounbinding was visualized by subsequent staining with phalloidin-A647.
To determine if unlabeled phalloidin can be photo-unbound as well, another assay was performed at which unlabeled phalloidin was used in excess. For this purpose primary staining was done with unlabeled phalloidin and phalloidin-A488 at a ratio of 4 (unlabeled):1 (labeled). A small portion of labeled phalloidin was necessary to set the focus for bleaching. Again actin filaments were excited by 1PE and 2PE and subsequently re-stained with phalloidin-A647.

2.7. Data Acquisition

Data acquisition was done by a four step procedure:

1. Illumination by one or two photon excitation to induce photounbinding
2. Acquisition of a fluorescence image of the bleached and surrounding area
3. Reincubation with a differently labeled binding partner to visualize photounbinding
4. Acquisition of a fluorescence image to measure rebinding of the differently labeled binding partner

1) Square patches were bleached on the labeled CaM/CKII coated surface (a. CaM/CKII) or onto stained actin filaments (b. phalloidin/actin) using a Laser Scanning Microscope (Zeiss LSM 510 confocal) to induce photounbinding. Bleaching was done by one (488 and 489 nm) and two (800 nm) photon excitation. To avoid rebinding of unbound CaM or phalloidin, the bleaching step was done in relatively high volumes of buffer solution. Following light induced dissociation free binding sites are available.

2) Square patches and the surrounding area were imaged (2 channel image: red and green). Green color displayed corresponds to Alexa Fluor 488; red color to Alexa Fluor 647. Imaging was done with very low intensities to avoid unwanted bleaching/unbinding.

3) The sample was incubated with a differently labeled binding partner to visualize free binding sites. 4. Another image was taken (red and green channel). Photounbinding was measured and quantified by determining the amount of rebound CaM (a. CaM/CKII) or phalloidin (b. phalloidin/actin).

2.8. Computer based data analysis

The obtained data of the photounbinding assay (one photon excitation) was analyzed with a custom-made computer software.

Below (fig. 7), a scheme of a bleaching pattern after one photon excitation, (CKII/CaM-A488 coated surface) is shown. Based on lines 1, 2 and 3, intensity values of an average of 30 pixels were calculated.

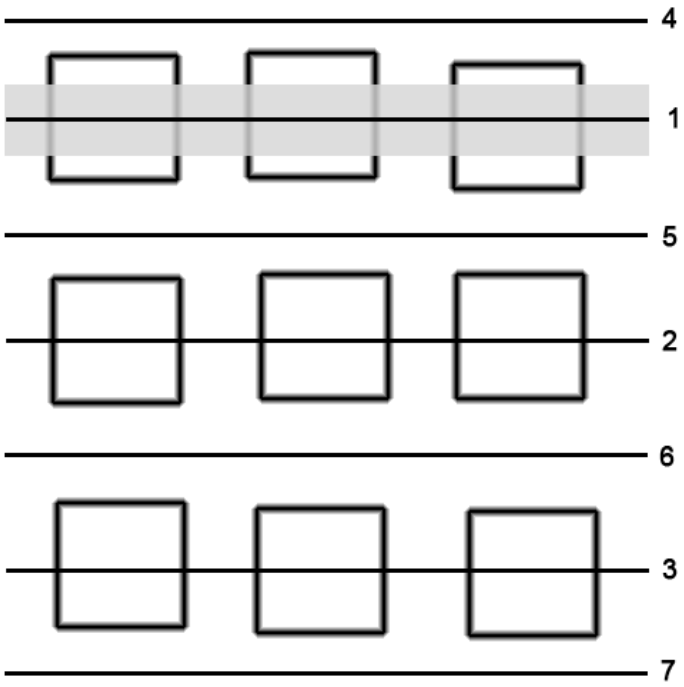


Fig. 7: bleaching pattern (one photon excitation); increasing laser intensities from top left to bottom right

Lines 4, 5, 6 and 7 served as reference-values for background levels. Because the glass bottom petri dishes could not be aligned to be absolutely even on the microscope stage, background levels varied between the lines. Hence, the average value of the background lines adjacent to lines 1, 2 or 3 were subtracted from the corresponding intensity values.

The curves were smoothed and analyzed.

Analysis of rebinding pattern:

X1= : > (line1 - Mean Line 4+5)/RMax$_1$
> (line1 - Mean Line 5+6)/RMax$_1$
> (line1 - Mean Line 6+7)/Rmax$_1$

Analysis of bleaching pattern:

X2= > (Mean Line 4+5 - line1)/Mean Line 4+5
> (Mean Line 5+6 - line2)/Mean Line 5+6
> (Mean Line 6+7 - line3)/Mean Line 6+7

For further analysis, the values were normalized and plotted in Origin.

Normalization:

CaM-A488 fluorescence (after bleaching):

- background values were subtracted (*X2 - background*)
- values have been normalized:
  (*Ymax.* = highest value (remaining CaM-A488 fluorescence) of all tested CKII peptides after background subtraction)

$$\frac{1}{Ymax.} * (X2 - background)$$

Rebinding pattern (CaM-A647 fluorescence) – after CaM-A647 reincubation:

$$X1/(Rmax - unspecific\ binding)$$

Rmax: mean values of all control measurements within a single measurement series (same crosslinker, peptide, CaM concentrations; same incubation times, temperatures; same laser settings)
Control measurements: Peptide coated cover-glasses were blocked (blocking buffer, 20 minutes, room temperature) and incubated with CaM-A647. The control measurements reflect the maximal rebinding values on the CKII coated surface.

Unspecific binding: Unspecific binding of CaM-A647 to the CKII coated glass surface (see results).

Analysis of two photon excitation induced unbinding was done manually.
The fluorescence intensities were obtained via surface plots of the CaM coated surface – surface plot green channel: CaM-A488 after bleaching, surface plot red channel: CaM-A647 after reincubation (ImageJ). Percentages of rebinding (CaM-A647 fluorescence in the bleached patches) and unbinding/bleaching (remaining CaM-A488 fluorescence in the illuminated patches) were calculated based on these surface plots. Graphs were generated in Origin 7.5.

3. Results

For analysis of the photounbinding of fluorescently labeled Calmodulin from a set of CKII peptides, an assay was developed and optimized. CKII peptides were coupled onto a silanized cover-glass using a crosslinker [SM(PEG)$_8$] of 39.25 Å length. Immobilization of the sample is important to ensure that no protein resources are unintentionally exchanged during the experiment. The relatively long crosslinker serves as a 'spacer' to minimize interactions of the CKII peptides or CaM with the glass surface. The CKII coated cover-glass is well suited to perform light induced unbinding and rebinding studies as the surface can be illuminated and imaged using a confocal microscope. After illuminating a defined square patch, the photounbinding effect was visualized and measured by re-binding a second CaM with a different fluorescence. The goal was to investigate whether the CKII peptides remained functional for re-association following the photo-induced dissociation. The key feature of the experimental setup was to see whether a vacated binding site is made available for rebinding after photounbinding, and a binding site occupied by a molecule carrying a bleached fluorophore is not. In this way the loss of fluorescence could then be allotted to bleaching or unbinding in a quantitative manner.

Since no suitable protocol was available, the optimal concentrations and incubation times to coat cover-glasses with the SM(PEG)$_8$ crosslinker, CKII peptides and Calmodulin, were determined experimentally. To assure minimal unspecific binding and strong fluorescent signals a homogenous and saturating coating with the SM(PEG)$_8$ crosslinker/CKII peptides and CaM was crucial.

Details of the sample preparation and individual steps of photounbinding experiments are described in the *Material and Methods* section of this thesis.

3. 1. Fluorescently labeled Calmodulin undergoes unbinding from CKII peptides

As described above, CKII peptide coated cover-glasses were incubated with CaM-A488 for several hours, to saturate all binding sites (for details see *Material and Methods*).

Nine square patches with increasing laser intensities were “bleached” onto the CaM-A488 - peptide surface with an Argon 489 nm laser. The laser intensity was varied from patch to patch, ranging from a few μW to several mW (see below). An example of a bleaching-pattern on the CaM-A488 surface can be seen in fig. 8. The imaging of the bleaching pattern was performed using very low laser intensities (significantly below the lowest bleaching intensity) to avoid undesirable bleaching or photounbinding.

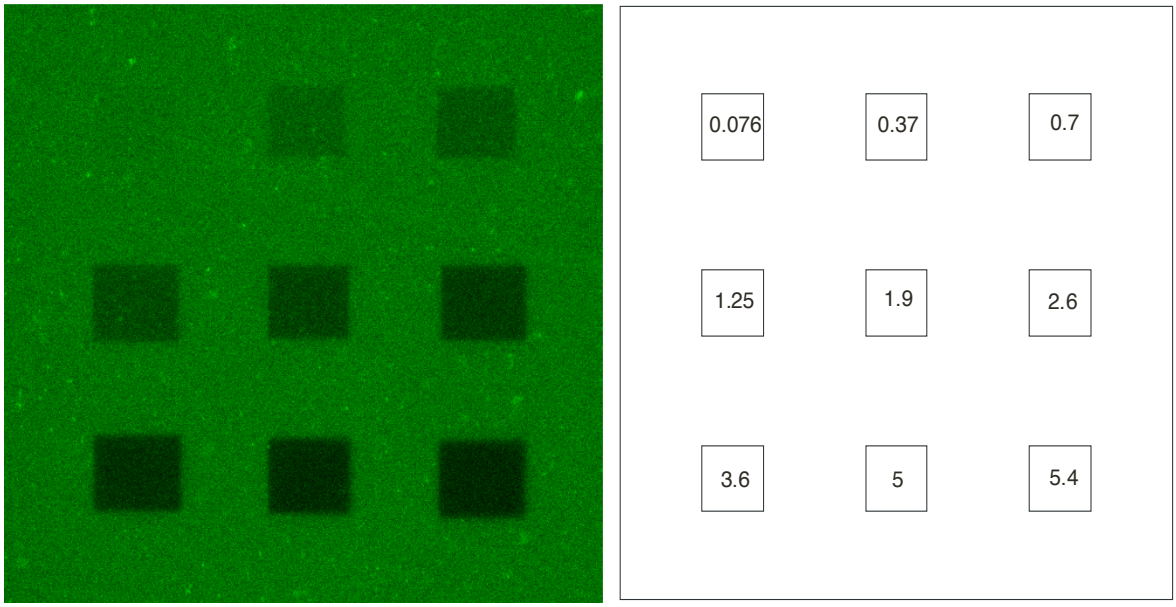
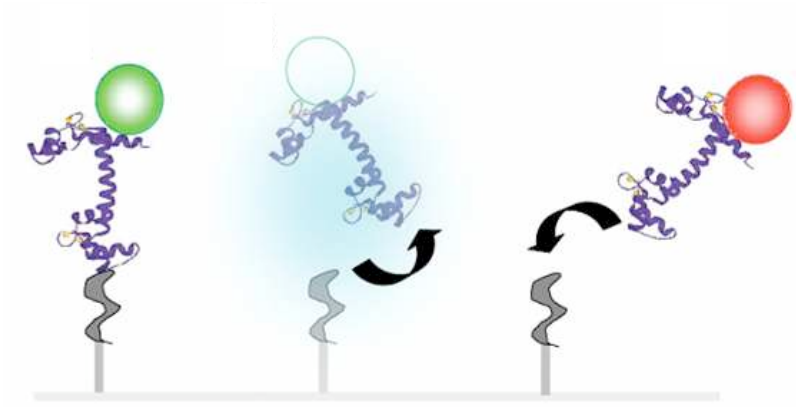


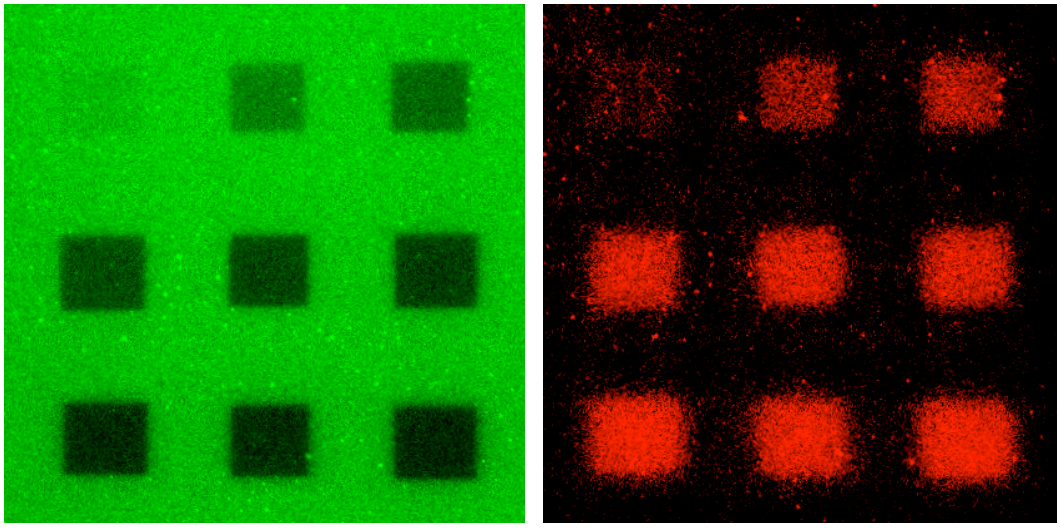
Fig. 8: (left) example of a bleaching pattern applied on a CaM-A488/CKII coated cover-glass; the peptide/Calmodulin coated surface was illuminated with an Argon 489 nm laser with increasing intensities from top left to bottom right (76µW – 5.4mW); (right) corresponding bleaching intensities in mW (1PE, 489 nm)

To visualize photounbinding and distinguish it from photobleaching, the illuminated CaM-A488/CKII surface was incubated with a differently labeled CaM for 40 minutes, rinsed with buffer solution (3x) and imaged immediately afterwards. The amount of photounbound CaM was quantified indirectly by parallel calibration measurements of the binding of CaM-A647 to the peptide only (i.e. determining the maximum CaM-A647 fluorescence possible in this experimental setting) while using the same CaM-A647concentration and incubation time (40 minutes) as for the CaM-A647 rebinding experiments. Again, imaging for photounbinding analysis was done with laser intensities significantly below the ‘bleaching’ threshold to avoid additional photo-bleaching and photounbinding of labeled Calmodulin after the experiment.

A clear rebinding pattern in the red imaging channel could be observed after CaM-A647 reincubation [fig. 9 (A)] which is clearly correlated to the ‘bleaching’ pattern of CaM-A488 in the green imaging channel.



A



B

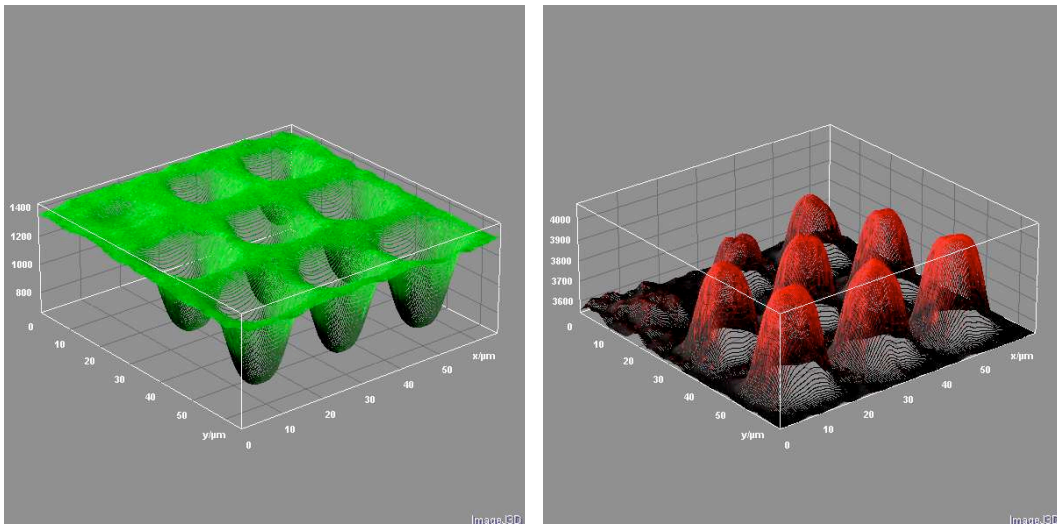


Fig. 9: (A) left: bleaching pattern (CaM-A488 fluorescence), right: corresponding rebinding pattern (CaM-A647 fluorescence); bleaching: 76 µW – 5.4 mW as indicated in Fig. 8.
(B) left: 3D surface plot (CaM-A488 fluorescence); right: 3D surface plot (CaM-A647 fluorescence)

The CaM-A647 rebinding pattern (fig. 9) clearly shows that laser illumination leads to unbinding of labeled CaM since the CaM-A647 only bound to peptides within the illuminated squares. As apparent in fig. 9 and fig. 10, a strong correlation between laser intensities, loss of fluorescence, and the amount of rebinding was observed. Also, photounbinding of CaM-A488 is stronger for higher laser intensities, yet laser intensities of <100 μW (inducing only a weak loss of fluorescence) already resulted in a clearly detectable CaM-A647 rebinding pattern as well. This suggests that photounbinding of labeled CaM already occurs at very low laser intensities, such as those commonly used in confocal microscopy, and is hard to distinguish from photobleaching in a typical imaging setup.

The experiment demonstrates that in addition to antibody-antigen bonding (Heinze et al. 2009), protein-peptide interactions (CaM-A488/CKII peptides) are also affected by laser illumination.

3.2. Photounbinding of labeled CaM from CKII peptides is dependent on the dissociation constant

To learn more about the photounbinding mechanisms, we investigate whether photounbinding shows a dependence on the dissociation constant of the molecular system. The Calmodulin-CKII system allows for photounbinding studies under different dissociation constants without changing the molecular system.

Therefore five CKII peptides with different binding affinities to Calmodulin ─ spanning three orders of magnitude of dissociation constants ─ were synthesized (based on sequences published by Waxham et. al. 1998) to quantify the dependence of photounbinding on the K_d.

One-photon induced unbinding of labeled Calmodulin was measured with the following peptides: CKII(290-312), CKII(291-312), CKII(292-312), CKII(293-312) and CKII(294-312). The dissociation constant of the CaM-various CKII peptides interactions decreases with increasing length of the CKII peptide. Table 2 summarizes the K_ds of CaM and the different CKII peptides. The five selected peptides for the following unbinding experiments are highlighted.

Peptide	Sequence 290 295 310	$k_{on} \times 10^7$	$k_{off} \times 10^{-5}$ k_1	k_2	$K_d \times 10^{-13}$	ΔG^0
		$M^{-1}s^{-1}$	s^{-1}		M	$kcal/M$
CKII(296–312)	RRK–TRN	13	26,000		20,000	−11.7
CKII(295–312)	**A**RRK–TRN	15	25,000		17,000	−11.8
CKII(294–312)	**NA**RRK–TRN	7	400		570	−13.8
CKII(293–312)	**FNA**RRK–TRN	12	20		17	−15.9
CKII(292–312)	**KFNA**RRK–TRN	21	10	130 (16)	5	−16.7
CKII(291–312)	**KKFNA**RRK–TRN	32	7	110 (13)	2	−17.1
CKII(290–312)	**LKKFNA**RRK–TRN	27	9	85 (21)	3	−16.9
CKII(296–312)*	AAA–TRN	0.6	7,000		120,000	−10.7
CKII(293–312)*	**FNA**AAA–TRN	1.9	10,000		52,000	−11.2

Table 2 - taken from (Waxham et al. 1998): dissociation constants, on- and off-rates of CKII peptides

For the photounbinding assay, times between rinsing after CaM-A647 incubation and imaging were kept short (below two minutes). This is crucial for minimizing measurement errors that would arise from any CaM that diffuses into the buffer solution from the CKII coated surface.

To obtain comparable results, reactions with different CKII-peptides were performed simultaneously under the exact same conditions (peptide and CaM-concentrations, incubation time, illumination and imaging settings) for a series of measurements.

For quantitative analysis CaM-A647 rebinding values were background-corrected as well as corrected for the fraction of fluorescence loss that occurs due to higher off-rates of the CKII peptide rather than due to bleaching/unbinding. Especially data based on peptides with higher off rates [e.g. CKII (294-312)] were otherwise slightly biased as CaM may come off from the peptide without illumination before the experiment was finished. For the CKII(290, 291, 292 – 312) peptides with lower off rates, bias due to spontaneous dissociation was not observed. For further details on this see chapter 3.2.1 (Mathematical correction of CaM-A647 rebinding values).

The data (fig. 10 and fig. 11) clearly show that the tested CKII peptides were affected by photounbinding. Photounbinding decreases with increasing dissociation constants of the molecular system.

As (exclusive) photobleaching would be the same for all A488-CaM for a certain laser power, the trend in fluorescence loss and rebinding observed here most likely arises from the different dissociation constants of the CKII peptides. This assumption is supported by the fact that the amount of CaM-A647 rebinding still correlates with the decrease of fluorescence after bleaching. The lowest amount of light induced unbinding was found with the CKII(294-312) peptide. This Calmodulin-peptide interaction has the highest

dissociation constant within the tested CKII peptides. The CKII(290-312) peptide, which has the lowest dissociation constant, was most affected by photounbinding.

A

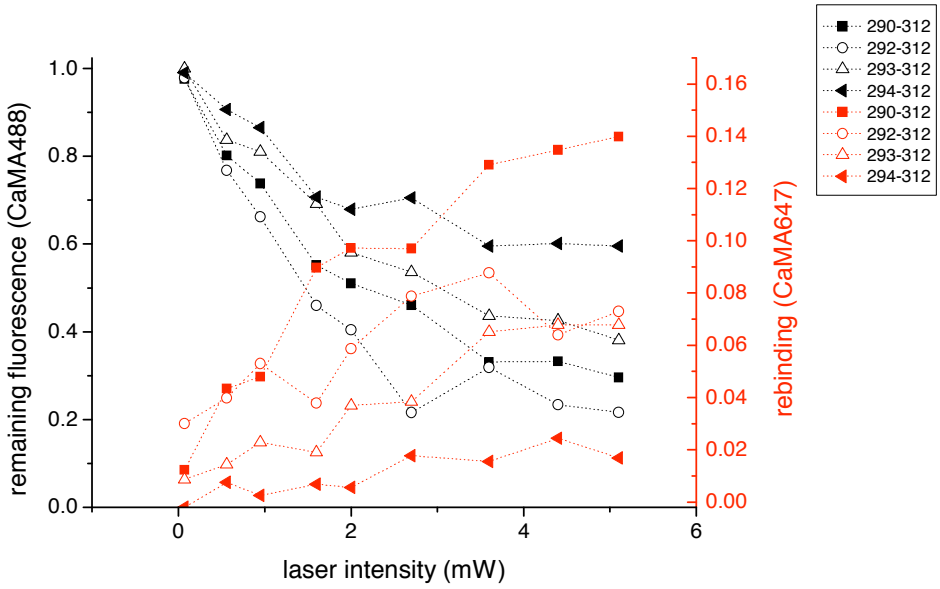


Fig. 10 (A): one photon excitation induced unbinding of labeled CaM from different CKII peptides; remaining CaM-A488 fluorescence in the bleached patches (black lines); CaM-A647 fluorescence within the bleached patches after CaM-A647 incubation (red lines);

B

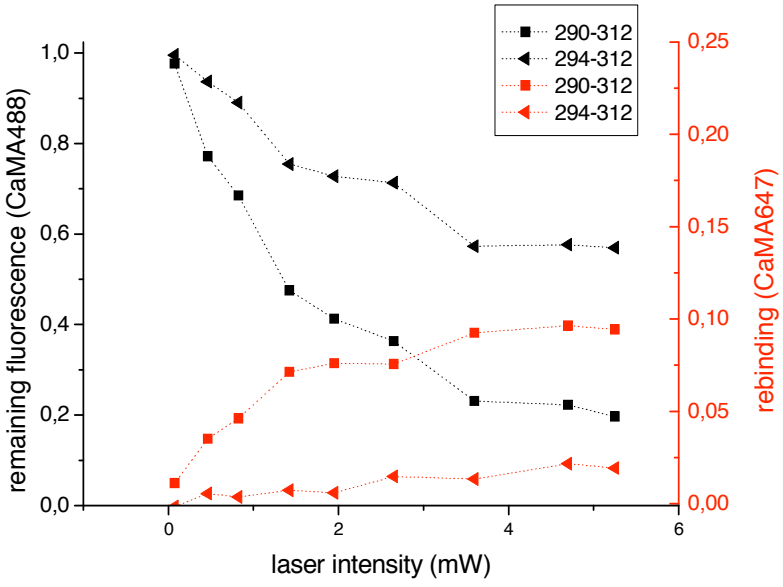


Fig. 10 (B): bleaching/photounbinding and rebinding curves of labeled CaM to the peptides CKII(290-312) and CKII(294-312) (mean values of two measurements).

The CKII(294-312) peptide which shows the lowest CaM rebinding values is also the shortest in length, and has a K_d which is significantly higher than the K_d of the CKII(293-,

292-, 291- and 290-312) peptides [at a factor of 33.5 compared to CKII(293-312) and at a factor of ~ 114 to 285 compared to CKII(290,291,292-312)], whereas the K_d of CKII(293-312) differs from CKII(290-312) only by a factor of 5.7.

The differences concerning rebinding levels between high and low affinity peptides are illustrated in fig. 11, which shows the mean values of three measurements. The trend of decreasing photounbinding with increasing dissociation constant from CKII(290-312) to CKII(292-312), CKII(293-312) and CKII(294-312) is illustrated in fig. 11 as well.

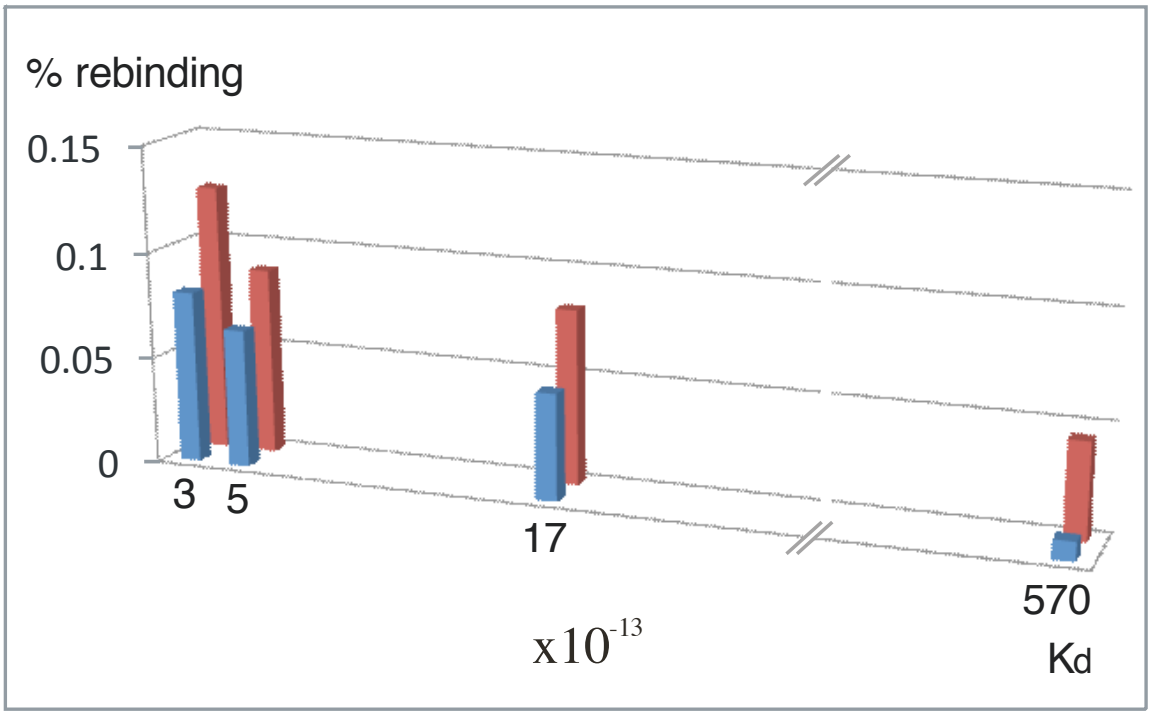


Fig. 11: amount of rebinding to various CKII peptides; bleached with 3.6 mW, one iteration (blue); bleached with 3.6mW, two iterations (red); diagram is based on corrected rebinding values (chapter 3.2.1.) and shows mean values of three measurements.

Due to the limitation that 100% laser intensity (~5.5-6 mW) was not sufficient to completely bleach/unbind all of the CaM-A488 in the illuminated area, bleaching was done with two iterations as well. Fig. 12 illustrates that the bleaching curve reaches saturation at ~5 mW for the CaM-A488/CKII(290-312) interaction when bleached with 2 iterations. As one can see in fig. 11, CaM rebinding to the CKII(294-312) peptide strongly increased (by a factor of 5) when a second bleaching step was done. This shows that there was still a lot of CaM-A488 that was bound to the CKII(294-312) coated surface after bleaching for one-iteration (3.6 mW) that could be dissociated. In contrast, the rebinding value of CaM-A647 to high affinity peptides like CKII(290-312) increased only by 30%,

when bleaching was done with two iterations. This finding indicates that there a higher laser power is required to dissociate lower affinity interactions such as CaM-A488/CKII(294-312). The maximal photounbinding values are approximately 80 % higher for the CKII(294-312) peptide when the absolute amount of light on the sample is doubled.

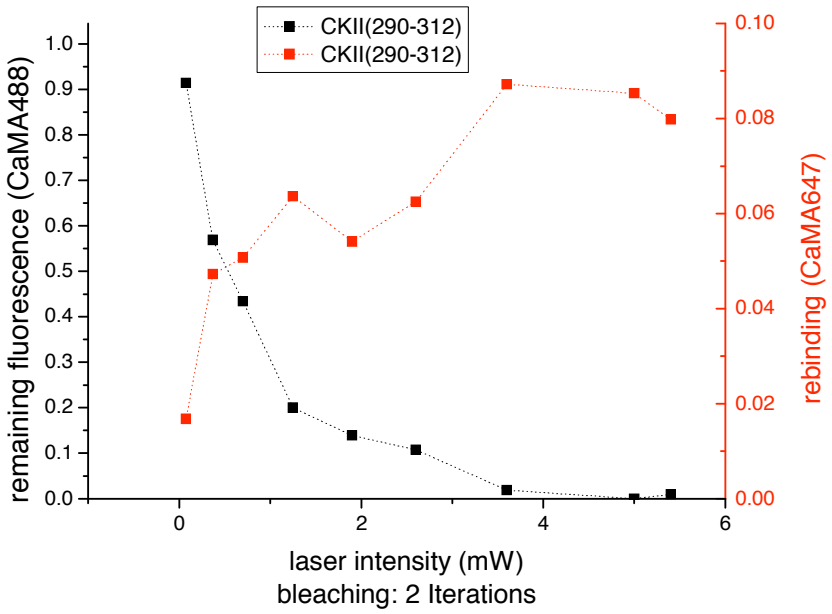


Fig. 12: bleaching/unbinding (black) and rebinding (red) curves on a CKII(290-312) coated glass surface for labeled CaM; (bleaching curve reaches saturation at ~4mW, bleaching: 489 nm, 2 iterations)

In general, laser intensities below 2 mW are sufficient to bleach or unbind the majority of CaM-A488. The amount of rebinding increases and draws near saturation somewhere above 5 mW (2 iterations) since maximal bleaching of the CaM-A488/CKII(290-312) peptide coated surface seems to already be reached at these laser intensities.

It is not clear yet though why photounbinding is more likely for stronger initial binding constants. Based on these studies we can only speculate that it may be related to the rigidity of the proteins/peptides involved.

3.2.1. Mathematical correction of CaM-A647 rebinding values

As already mentioned, it is important in the data analysis to take into consideration that the CKII - peptides have different off rates (see table 2). The measurements of the CaM-A647 rebinding intensities were done approximately two minutes after CaM-A647 incubation. This time was necessary to rinse the cover-glasses and to do final imaging. Due to the different off-rates of the Calmodulin-CKII interactions there are aberrations from the real rebinding intensities, as a certain amount of CaM-A647 is lost through spontaneous dissociation depending on the off rate of the respective peptide. These aberrations were corrected for by calculating the amount of CaM that was lost from the peptide coated surface through diffusion. The CaM-CKII off rates, previously measured by Waxham and colleagues, were found to show a 2-4 fold decrease when the temperature was lowered to 4°C from room temperature (Waxham et al. 1998). For the photounbinding assay, the CaM/CKII-peptide coated surface was rinsed and covered with 3-4 ml of CaM-buffer at a temperature of 4°C. Considering a slight temperature increase of the CaM-buffer (the temperature of a 3-4 ml CaM buffer solution would increase slightly when kept at room temperature for 1-2 minutes), the measured results were corrected by for assuming only a 2 fold decrease of the off rates. In this way the corrected data contain a conservative assumption of the error rate for the obtained rebinding intensities. Since the real rebinding intensities are likely to be slightly lower [especially in the case of the CKII(294-312) peptide coated surface] than the corrected values, the dependence on the dissociation constant might be slightly underestimated. It follows that the Kd-dependence may be stronger than that shown in fig. 10 and fig. 11. This should however not be crucial, as the differences between the corrected rebinding values and the original measured data is relatively small. The peptides CKII(290, 291, 292, 293 – 312) are hardly affected by the measurement error due to their small off rates (error < 1.5 %). The rebinding values on the CKII(294-312) peptide coated surface were shifted noticeable (~ 20%) due to an off rate of 0.002 s^{-1}. In summary there were slight changes in the rebinding values after correction but the trend with respect to the amount of photounbinding did not change significantly.

The amount of CaM-A647 that was lost through diffusion was calculated based on the off rates of various CKII peptides (Waxham et al. 1998). The respective photounbinding values corrected using the relation (Baumgarth et al. 2005):

$$koff = -d\left[\ln\left(\frac{PN}{PN0}\right)\right]/dt, \quad \text{(eq. 1)}$$

where koff is the off rate, PN the concentration of CaM/CKII at time *t* (in our case 2 minutes), and PN0 the concentration of CaM/CKII at *t*=0. PN0 was set to 100 % so that PN could be obtained as the percentage of the remaining CaM/CKII. The corrected rebinding value is finally calculated by the measured rebinding value multiplied by 100/PN.

For a normalized *PN0=1* the table below shows the fluorescent fractions (*PN*) that have to be considered for the rebinding calculation

PN	*t(sec)*	*off rates*	*CKII peptides*
0.787	120	0.002	294-312
0.988	120	0.0001	293-312
0.994	120	0.00005	292-312
0.996	120	0.000035	291-312
0.995	120	0.000045	290-312

table 3: corrected fluorescent fractions (PN), calculation was done according to the procedure described above

Example calculation:

Calculation of rebinding values and data correction for CKII(293-312)/CaM-A647 interaction:

$$\frac{measured\ fluorescence\ (CaM\ A647\ rebinding)}{max.rebinding\ control - background\ fluorescence} * 100 = \%\ rebinding$$

The measured fluorescence (CaM-A647 rebinding) is discussed in the *Material & Methods* and *Data Analysis* sections.

In this example the measured CaM-A647 fluorescence is taken to be 25.21951 gray values. Note: bleaching before reincubation was done at 76 μW.

The fraction of CaM A647 that rebinds is in this case:

$$\frac{25.21951}{3178 - 1340} = 0.01372$$

or

$$0.01372 * 100 = 1.372\%\ CaM\ A647\ rebinding\ (bleaching: 76\mu W)$$

Correction this for the CKII(290-312) peptide coated surface one has:

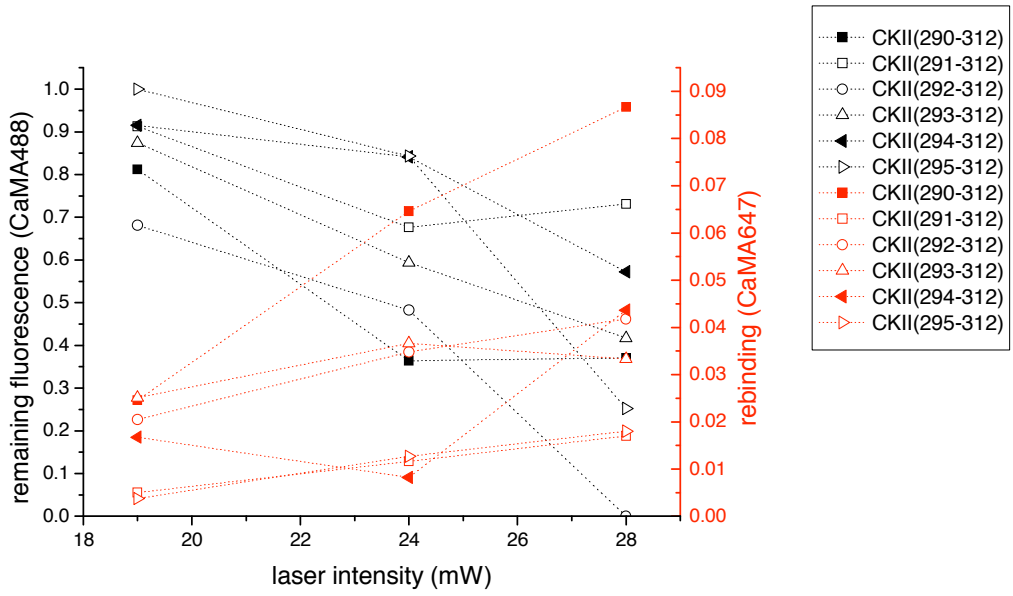
$$1.373\% * \frac{100}{98.8} = 1.39\%$$

3.3. Photounbinding of labeled Calmodulin by two-photon excitation

As described above, labeled CaM can be unbound from its binding partner (CKII-peptides) by one photon excitation. Another question is whether photounbinding can also be observed after two photon excitation. For two photon exication (2PE) of the CKII – CaM-A488 coated surface a mode-locked Ti:Sa laser was used at a peak wavelength of 790 nm with laser intensities from 16 to 33 mW (inducing slight to strong loss of fluorescence). Finally photounbinding was visualized by subsequent rebinding of A647-CaM as described before.

A rebinding pattern after 2PE was clearly observable with all tested peptides (CKII(290, 291, 292, 293, 294, 295 – 312)). As observed after one photon excitation (1PE), labeled CaM can dissociate from its binding partner after 2PE with a strong dependence on the laser intensity. However a dependence of photounbinding on the dissociation constant, which was clearly seen after one photon excitation, was not observed with 2PE (fig. 13). A possible explanation for this could be some technical difficulties encountered during the two photon measurements. Due to properties of the two photon microscope, minimal motions of the sample were possible. For the small two-photon excitation focus minimal changes in the focus-height could already have a dramatic effect on the measured fluorescence intensities as the fluorescence would come from a protein monolayer. This could explain the variation of measured rebinding values between the different measurement series that we obtained.

A



B

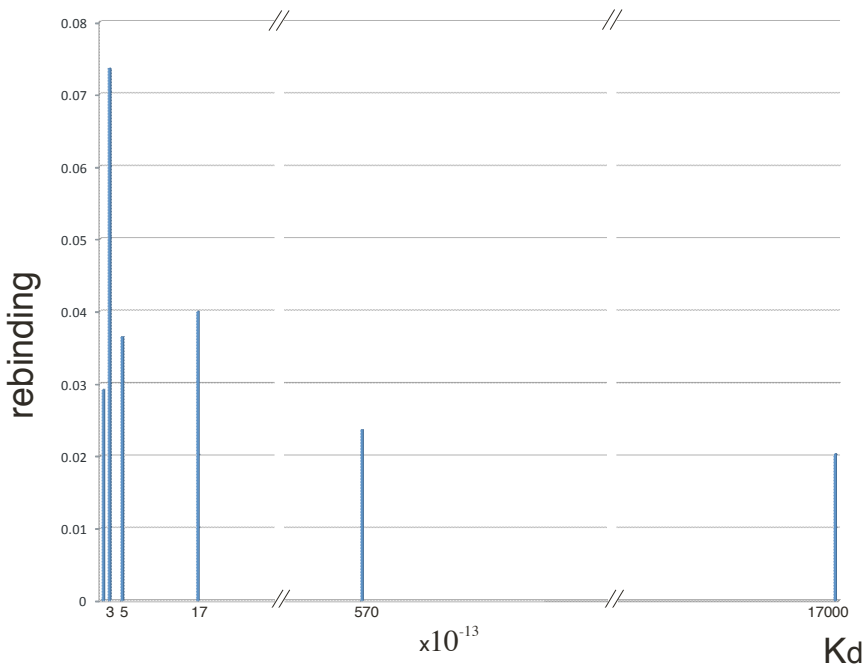


Fig. 13: photounbinding after 2PE; (A) remaining CaM-A488 fluorescence after bleaching (black lines); rebinding of CaM-A647 to bleached areas (red lines); single measurement;
(B) CaM-A647 rebinding values; bleaching: 24mW; mean values of 3 measurements

3.4. Photounbinding requires an absorbing or fluorescent label

One step towards the mechanism underlying photounbinding is to investigate whether a fluorescent label is required to induce photounbinding. Therefore we have performed the same experiment as described before on non-fluorescently labeled CaM. Unlabeled CaM was incubated on CKII(290-312) peptides and photounbinding assays with one and two photon excitation were performed. To ensure that unlabeled CaM was within the focus during bleaching, green fluorescent nanobeads were used in high dilution as an additive to facilitate focusing on the confocal microscope (fig. 14). The coated cover-glasses were incubated with small fluorescent beads (40 nm diameter) for several minutes until the beads attached onto the glass surface and could be brought into focus. A dilution was chosen for which only few beads were visible (and well distinguishable) in the field of view– just enough to adjust the focus plane. This would avoid possible side effects.

For incubation of the CKII(290-312) coated surface a CaM concentration of 60.4 μM was used. This would guarantee complete saturation of all free binding sites.

We found that one and two photon excitation did not induce photounbinding, based on the fact that no rebinding pattern corresponding to the illuminated square patch (dashed lines in fig. 11) of CaM-A647 could be seen. Only weak and homogeneously distributed unspecific binding of CaM-A647 to the peptide coated glass surface was observed. Based on these and previous findings (Heinze et al. 2009) it can be concluded that photounbinding requires a fluorescent (or at least absorbing) label to show photounbinding after 1PE or 2PE.

To ensure that the (invisible) unlabeled CaM was present on the glass surface and homogenously bound to the CKII(290-312) peptide coated cover-glass, we performed an anti-CaM (IgG) antibody staining in combination with an A568- anti-IgG as a secondary antibody. A fluorescent image of the stained Calmodulin-peptide coated surface next to a CaM-free area was taken (fig. 14, right). The two areas (stained and unstained) are clearly distinct and indicate the proper CaM coating of the experiment shown in fig. 14.

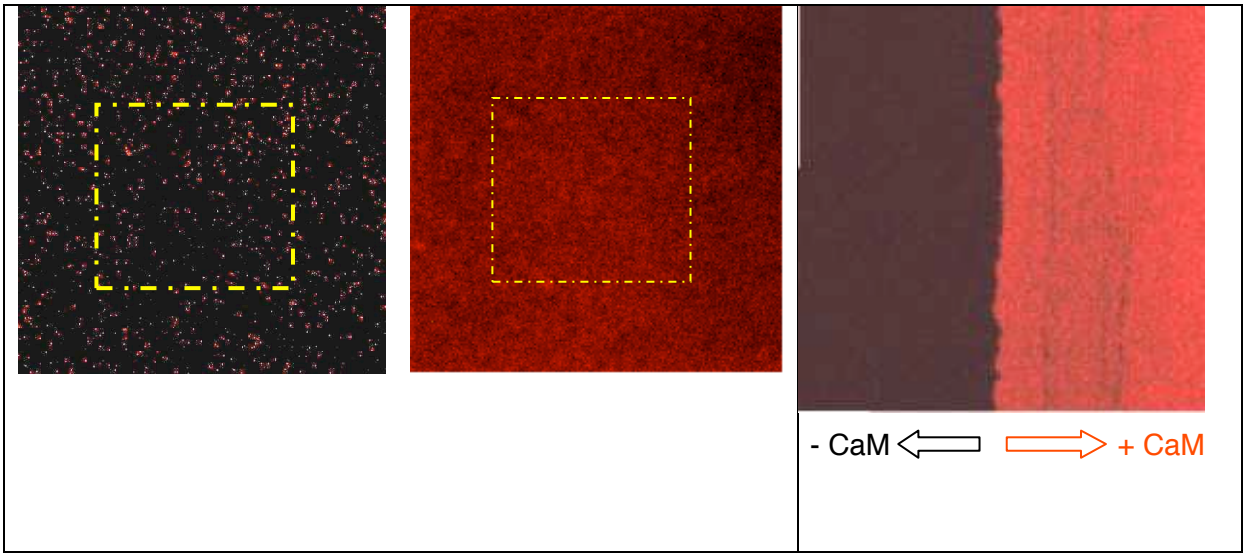


Fig. 14: unlabeled CaM – assay: (left) unlabeled CaM/CKII peptides surface; (middle) CaM/CKII surface after CaM-A647 reincubation (no rebinding pattern detectable); (right) staining of CaM/CKII coated surface

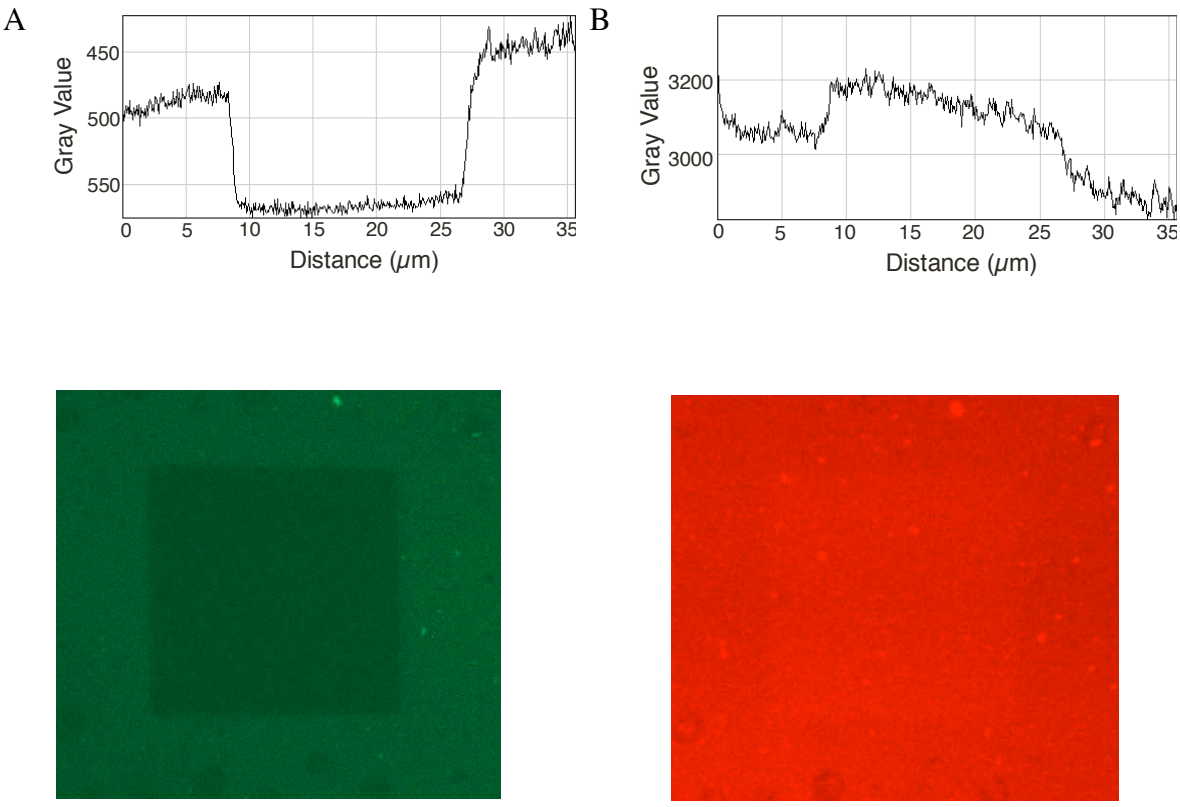
3.5. Photounbinding is directly linked to photobleaching

The lifetime of a fluorescent dye, when illuminated by two photon excitation, can be extended by adding the chemical stabilizer ascorbic acid. Ascorbic acid can prevent the bleaching pathway by its reducing properties (Dittrich et al. 2001).

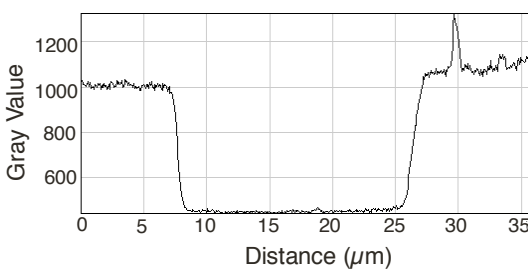
To determine if bleaching and photounbinding are linked or occur independently, a photounbinding assay was carried out, at which ascorbic acid was added before the photounbinding step at a concentration of 10mM with the pH adjusted to 7.2 by adding HCL. The idea behind the experiment is the following: If photounbinding and photobleaching would be independent processes, the fluorescence loss of the immobilized A488-CaM/CKII peptide could not be (fully) prevented by a stabilizer preventing photobleaching. However, if photo-bleaching is induced first (before photounbinding) we would see a two–fold fluorescence stabilization as reported by Dittrich et al. 2001 even for an immobilized assay where no dye resources can be exchanged during illumination.

According to the photounbinding assay protocol, CaM-A488 was incubated for several hours on a CKII(290-312) peptide coated cover-glass to saturate all free peptides. After incubation, the CaM-A488 surface was covered with the ascorbic acid CaM buffer and illuminated as described in section 3.3. Next, the buffer was removed, the surface was incubated with CaM-A647 for ~40 minutes, rinsed, and imaged immediately.

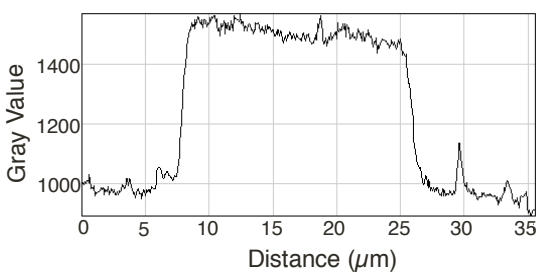
As a control, the same photounbinding assay was performed using CaM buffer without ascorbic acid. It turned out that both show a decrease in the CaM-A488 fluorescence after laser illumination, however the loss of fluorescence for the sample treated with ascorbic acid was significantly smaller than for the control. The difference in fluorescence loss is in agreement with the findings of Dittrich et al. 2001 who reported that bleaching of A488 could be reduced to up to 50% using the chemical stabilizer. The amount of CaM-A647 - rebinding was also reduced up to 50% when ascorbic acid was used. These differences concerning photounbinding and photo-bleaching are visualized in fig. 15. It can be concluded from the results that photounbinding is directly linked to photobleaching and most likely occurs (shortly) after photobleaching. However, it is important to stress that the 'laser power window' where photobleaching is observed without any signs of unbinding is rather small for all molecular systems studied here and previously.

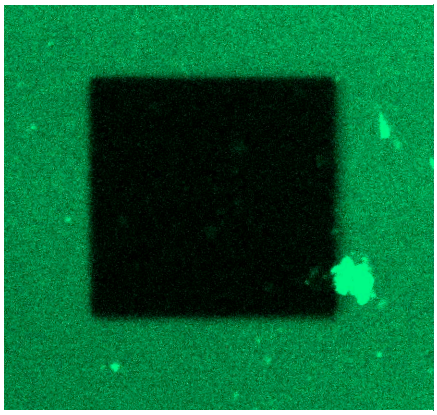


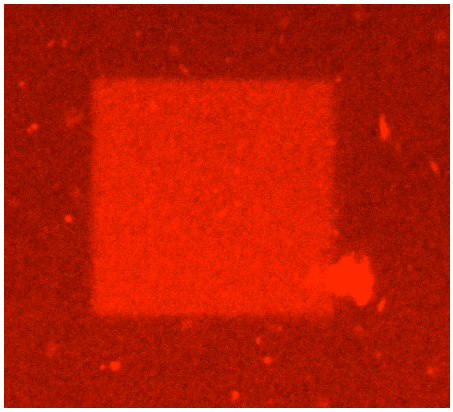
C


Gray Value
1200
1000
800
600
0
5
10
15
20
25
30
35
Distance (μm)

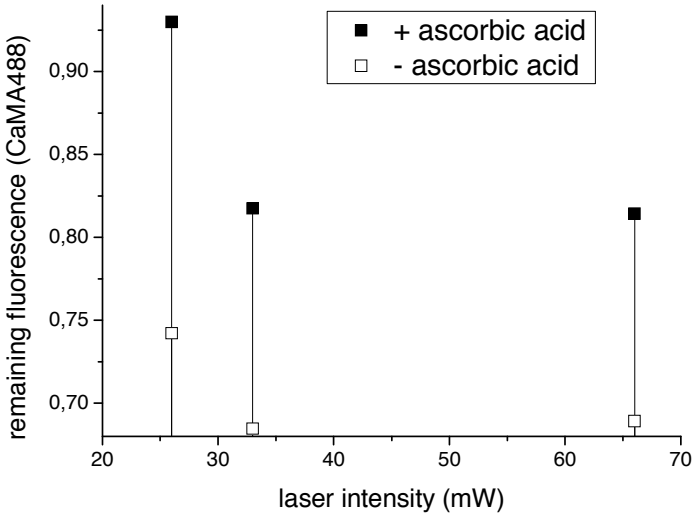
D


Gray Value
1400
1200
1000
0
5
10
15
20
25
30
35
Distance (μm)





E


+ ascorbic acid
- ascorbic acid
remaining fluorescence (CaMA488)
0,90
0,85
0,80
0,75
0,70
20
30
40
50
60
70
laser intensity (mW)

F

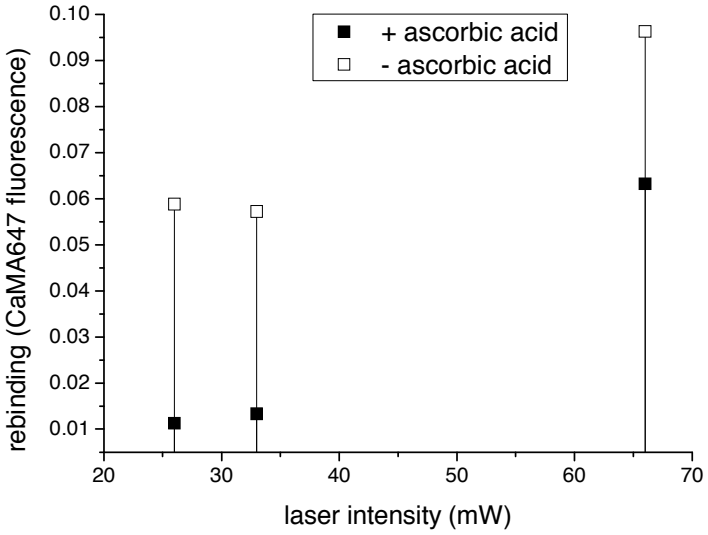


Fig. 15: surface plots of labeled CaM/CKII peptide coated surfaces after two photon bleaching (32 mW, 800 nm – bleaching was done with two iterations) and CaM-A647 reincubation; the bleached area starts at 8µm and ends at 27µm. (A) CaM-A488 (CaM buffer + 10 mM ascorbic acid) (B) CaM-A647 (CaM buffer + 10 mM ascorbic acid)
(C) control, CaM-A488 (CaM-buffer) (D) control, CaM-A647 (CaM-buffer)
(E) remaining CaM-A488 fluorescence after bleaching (with and without ascorbic acid)
(F) CaM-A647 rebinding to bleached areas (with and without ascorbic acid)

The graphs in figure 15 clearly show that rebinding of CaM-A647 was strongly decreased when bleaching was done in ascorbic acid containing buffer (E). As can be seen reduced bleaching is accompanied by decreased rebinding (F).

3.6. Photounbinding of CaM is not limited to a specific absorption wavelength or fluorophore

As reported for various antigen-antibody reactions in previous work (Heinze 2009), photounbinding is not limited to a specific absorption wavelength or fluorophore. Here, we test this hypothesis for the CaM/CKII peptide binding system under study to ensure that the observed unbinding effect is not a curiosity of a specific fluorescent label or laser wavelength.

As already shown, Calmodulin-A488 can be dissociated from CKII peptides by one and two photon excitation. To further characterize photounbinding, it would be important to know if the observed unbinding event is a consequence of specific properties of the Alexa Fluor 488 dye and/or of the specific wavelength that was used for bleaching (489 nm). We thus switched the fluorescent labels (A-488, A-647) used for unbinding and the rebinding step. To visualize photounbinding of CaM-A647, CaM-A488 was subsequently rebound to free CKII peptides in the bleached area.

For details about the photounbinding assay see *Material and Methods* (CaM-A647 was used for the first incubation step; CaM-A488 was used for reincubation; bleaching was done with 633 nm).

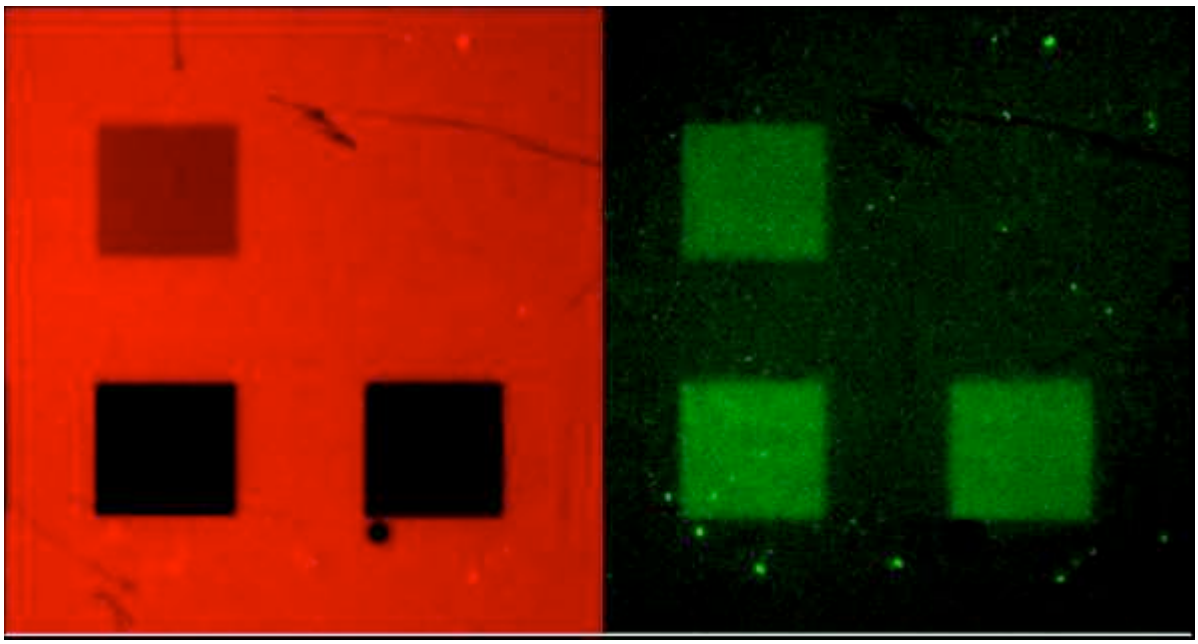


Fig. 16: photounbinding assay; CaM-A647 fluorescence (left); CaM-A488 fluorescence (right)

Fig. 16 shows that CaM-A647 can be photo-unbound from CKII(290-312). Photounbinding was visualized by CaM-A488 binding to free CKII peptides. A clear rebinding pattern that correlates with increasing bleaching intensities was observed.
The image shows that differently labeled Calmodulin (A488 or A647) can be dissociated from CKII peptides by light excitation and thereby strongly indicates that photounbinding is not restricted to certain properties of the conjugated dye or a specific bleaching-wavelength. Previous studies of antigen/antibody unbinding (Heinze et al. 2009) support these findings.

3.7. Key-Controls and calibration measurements

Some additional controls and calibration steps concerning the photounbinding assay on Calmodulin – CKII were done to allow for reliable data interpretation and quantification. These controls focus on unspecific binding and background, rebinding of identically labeled CaM and potential bias by fluorescent quenching.

3.7.1. Photounbinding does not occur for covalent bonds

In the following experiment we ask whether photounbinding occurs in the case of paraformaldehyde (PFA) fixation of the CaM/CKII peptide layer on the cover-glass. We performed two unbinding experiments: 1) PFA fixation of the CaM-A488/CKII surface *before* the bleaching step 2) PFA fixation *after* the bleaching step, but before rebinding. In case 1) it is interesting to know whether the covalent linkage between CaM-A488 and the CKII peptides due to the PFA fixation can be broken by light excitation. Case 2) serves as a control to ensure that the PFA chemistry does not interfere with the CaM-A647/CKII peptide rebinding.
In case 1) the CKII peptide was immobilized onto the cover-glass and incubated by a CaM-A488 solution. The sample was subsequently fixed with PFA (4%, 10 minutes at RT). After fixation the CaM-A488 protein surface was bleached and reincubated with CaM-A647, rinsed, and imaged immediately. As shown in fig. 17, no rebinding pattern was observed in the bleached area. For case 2), the PFA fixation step did not crosslink the

CKII(290-312)/CaM-A488 binding partners (see fig. 18), and rebinding occurred with a similar amount as the results obtained for no PFA fixation (chapter 3.1).

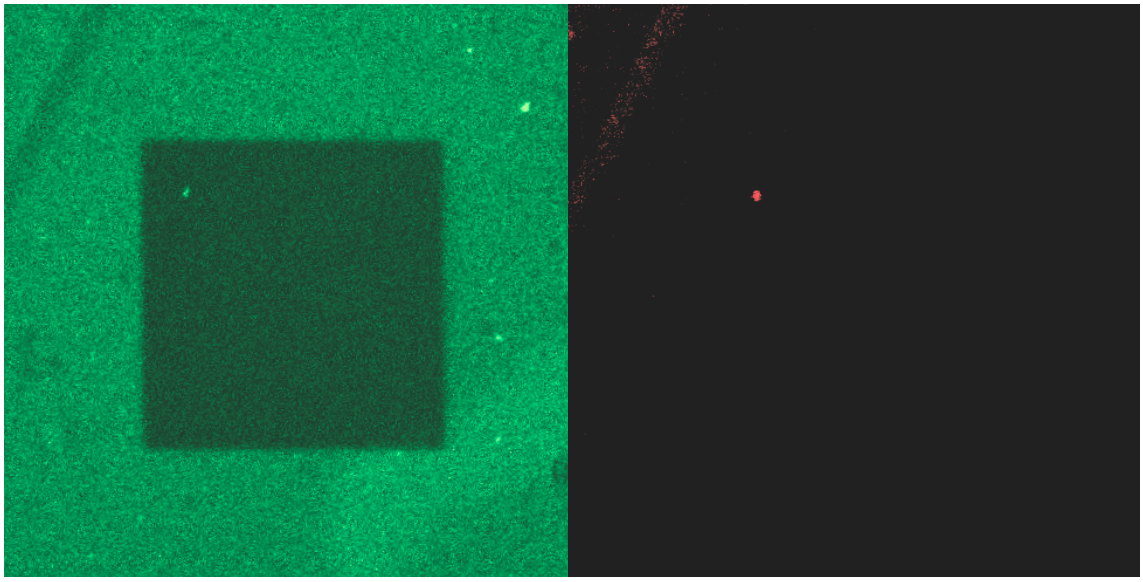


Fig. 17: photounbinding assay on a formaldehyde fixed CaM-A488/CKII(290-312) surface (bleaching: 2PE, 32 mW, 2 iterations, 800 nm); (left) CaM-A488 fluorescence, (right) CaM-A647 fluorescence

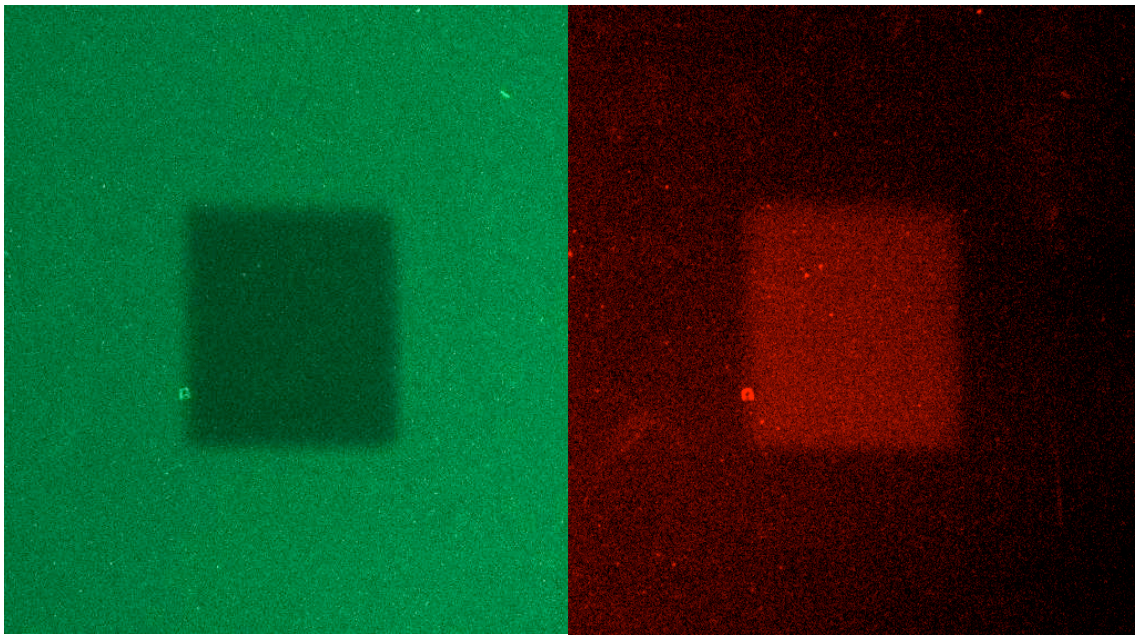


Fig. 18: positive control, PFA fixation was done before reincubation – no crosslinking of CaM-A488/CKII peptides (bleaching: 2PE, 31 mW, 800 nm)

As expected, the CaM-A488 cannot be unbound from CKII(290-312) after PFA fixation since the covalent linkage cannot be cracked by light excitation. Nevertheless, rebinding (as a measure of previous unbinding) can be observed in PFA fixed samples as long as the

fixation step did not crosslink the CaM-A488/CKII surface. One can conclude that PFA fixation did not interfere with rebinding following light induced unbinding. These findings are supported by another study which was done in PFA fixed cells and is described below (chapter 3.8).

3.7.2. Photounbinding does not occur with GFP-actin fusion proteins

The following experiment may reveal whether GFP can be dissociated from actin (in transgenic GFP-actin filaments) or GFP-actin complexes can be photounbound within actin filaments by light excitation.

The GFP actin fusion protein is very stable as GFP is attached to actin via a peptide bond. The contacts between neighboring protomers in an actin dimer have been previously described and are mainly from hydrogen bonds, salt bridges and van der Waals contacts (Kudryashov et al. 2005).

It is known that besides photobleaching such structures can be 'cut' by laser tools in so-called laser dissection routines (König et al. 2002). In both cases we would not expect to be able to selectively replace the bleached or destroyed filaments. However, it is interesting to investigate how the filaments react when using 'in between' laser intensities - i.e. intensities that are slightly beyond photo-bleaching, but not sufficient for laser dissection.

To test if light excitation induces the unbinding of GFP or of whole GFP-actin constructs, a small area of a transgenic GFP-actin cell was bleached. The cell was fixed, permeabilized (Triton X-100), and stained with anti-GFP-biotinylated/Streptavidin APC-Cy7.

As one can see in fig. 19 it looks like GFP could not be dissociated and actin filaments were not disrupted by light excitation. The actin filaments could not clearly be visualized with the anti-GFP-biotinylated/Streptavidin APC-Cy7 staining, as there was quite a high background staining [addition of BSA to the staining solution (1%) did not reduce the background staining noticeably]. However, there is also no decrease of the APC-Cy7 fluorescence in the bleached area, indicating that GFP-actin was not disrupted.

Most probably GFP could not have been dissociated from actin due to its strong covalent linkage. This is supported by the finding that formaldehyde fixed CaM-A488/CKII interactions could not be unbound as well (see above).

Also whole GFP-actin fusion proteins could not be dissociated within actin filaments. It is possible that a GFP-actin fusion protein fails to unbind from its binding partner, eventhough it was shown in previous work (Heinze et al. 2009) that GFP itself can be dissociated from antibodies by light excitation.

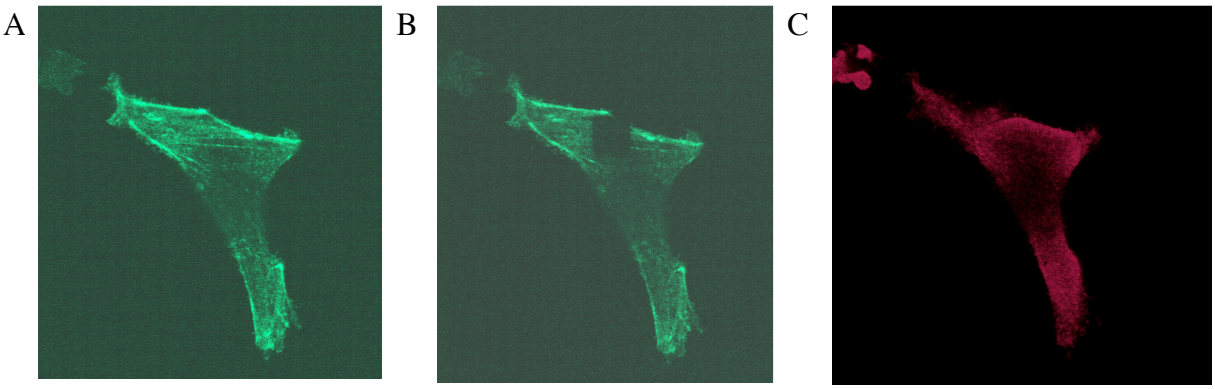


Fig. 19: (A) B16 transgenic GFP-actin cell, (B) a square was bleached onto GFP-actin filaments, (C) anti GFP staining

3.7.3. Rebinding of identically labeled Calmodulin

As an additional control and to further demonstrate the universality of the observed rebinding, an unbinding experiment was performed with identically labeled CaM. For both incubation and reincubation CaM-A488 was used and fluorescence in the bleached area was specifically restored to (a substantial part of) the A488 (fig. 20). This control also shows that the used CaM concentration in all reported experiments was sufficient to saturate the peptide coated surface in the first incubation step.

As shown in fig. 20, CaM-A488 could recover the fluorescence in the previously illuminated 'black' region by binding to free CKII peptides. Measurements of the mean intensity outside the bleached area before and after reincubation showed that the fluorescence intensity was almost equal in the non-bleached area indicating that the applied CaM concentration and incubation time leads to saturation of the CKII surface. The ~6 % higher fluorescence intensity outside the area of interest after reincubation is expected due to additional unspecific binding. The CaM-A488 intensity in the bleached region showed a clear increase in the A488 fluorescence, demonstrating rebinding of labeled CaM.

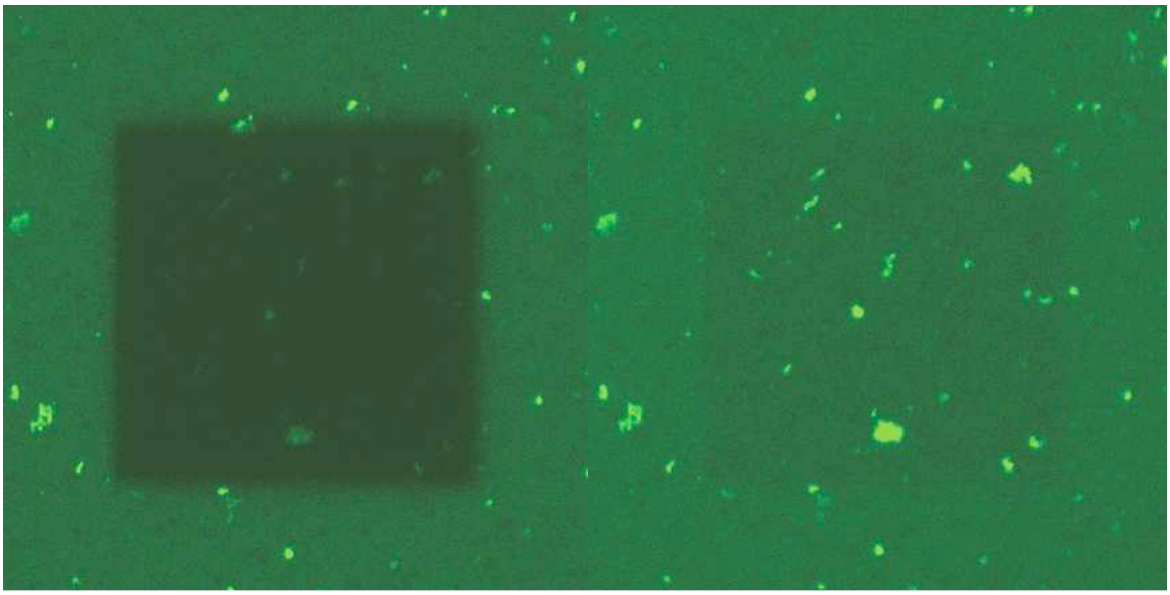


Fig. 20: photounbinding assay with identically labeled A488-CaM (illumination with 489 nm and 5.1 mW (1PE). The bright green spots in the image may be due to debris containing CaM-A488 which accidentally remained in the protein sample due to improper centrifugation. Unfortunately, this debris occasionally attach to the glass surface, however without disturbing the result and data analysis.

3.7.4. Potential fluorescence label interactions of Alexa488 and Alexa647

Two fluorescent dyes in close proximity may show different emission and absorption characteristics than the same dyes when isolated. One important effect is fluorescence quenching. Quenching refers to any process which decreases the fluorescence intensity of a given substance. A variety of processes are known to be able to induce quenching, such as excited state reactions, Förster resonance energy transfer (FRET), complex-formation, and collisional quenching. A high amount of fluorescence loss by quenching, particularly in the case of FRET, could bias quantitative analysis of our photounbinding experiments. Therefore it is important to uncover potential quenching effects between the used Alexa dyes (A488, A647).

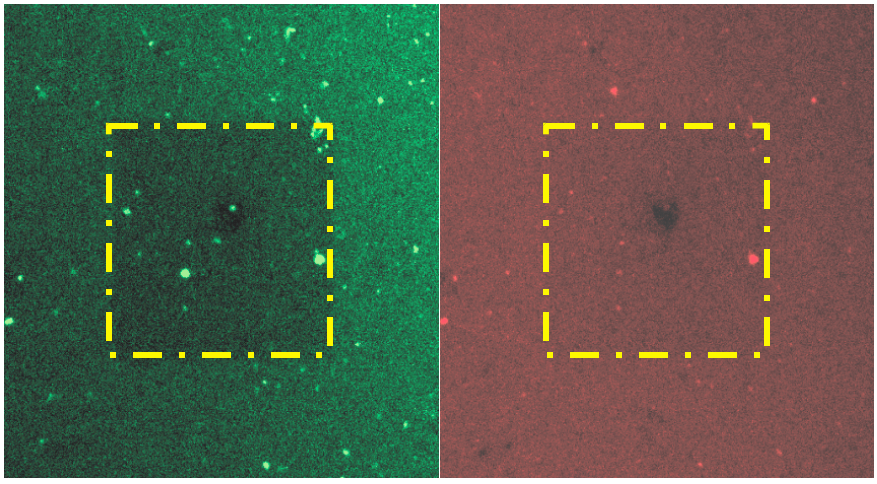
A CKII(290-312) coated cover-glass was incubated with equal concentrations of CaM-A488 and CaM-A647. Afterwards a square patch was laser illuminated with the blue (488 nm) or the red (633 nm) laser. It was examined whether the CaM-A488 or CaM-A647 fluorescence increases, either when losing its potential quencher by bleaching or by unbinding after light excitation. As shown in fig. 21 (A), no quenching was observed

when CaM-A488 was bleached/photo-unbound; the CaM-A647 fluorescence did not increase in the bleached area when blue laser illumination (488 nm) was used, meaning that there is no quenching effect biasing our experiments and assay. As the main measurement and quantification of photounbinding was based on CaM-A647 rebinding, the A-647 fluorescence was not affected by quenching and therefore quenching does not bias the measured rebinding values and has hardly any effect on this study.

However, as the experiments show, there is a potential bias when quenching is involved: When bleaching was done with the red laser (633 nm), a slight increase (< 5%) of the CaM-A488 fluorescence was observed, indicating that there is a slight quenching-effect [see fig. 21 (B), (C) and (D)]. The A647 label quenches a small amount of the A488 fluorescence. This means for the obtained results of the CaM/CKII unbinding study that some of the remaining A488 fluorescence in the bleached square patches may get quenched by rebound CaM-A647. Consequently, the amount of bleached/photo-unbound CaM-A488 is slightly overestimated (~5%), especially at high amounts of rebound CaM-A647.

It is obvious that the quenching bias would increase with increasing rebinding intensities. However, as rebinding intensities increase with increasing bleaching intensities the amount of remaining CaM-A488 decreases also. Thus the majority of CaM-A488 is already bleached/dissociated when quenching could significantly bias the measurements. The Quenching bias may be relevant when a high percentage of rebinding meets a high percentage of remaining A488-CaM fluorescence after illumination. This scenario is most likely at laser intensities of 2-3 mW. Therefore quenching might affect these data most, with an estimated maximum error <5%.

A



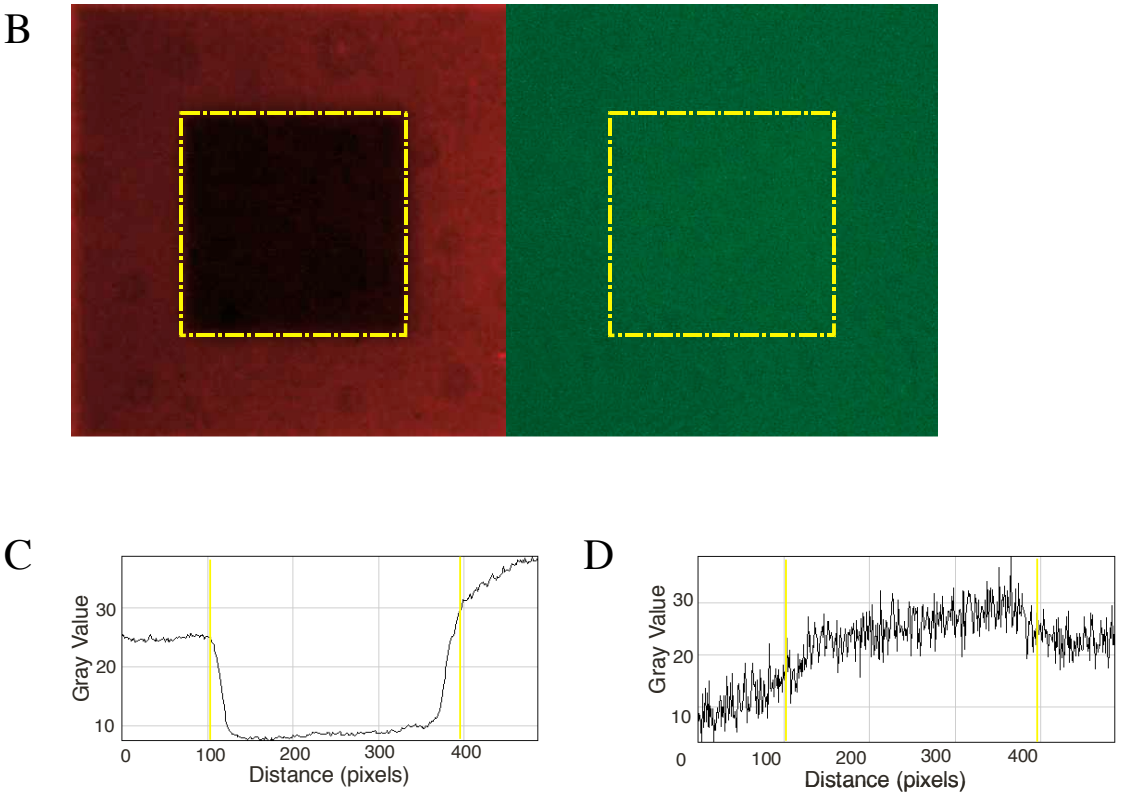


Fig. 21: quenching by label interaction of CaM-A488 and CaM-A647; (A) bleaching at 488 nm; (B) bleaching at 633 nm; (C), (D): surface blot of bleached + surrounding, non bleached - area, bleaching 633 nm; (C) CaM-A647 fluorescence; (D) CaM-A488 fluorescence)

3.7.5. Determination of unspecific binding of Calmodulin to the CKII peptide coated glass surface

The assay immobilized on a glass surface may imply the risk of unspecific binding of (labeled) CaM to the glass surface. This 'systematic background' has to be determined and controlled to minimize the effect on the sample since such CaM background fluorescence would decrease the dynamic range of the experiments when analyzing the unbinding fraction by the measure of molecules rebound. Therefore the amount of unspecific CaM-A647 rebinding was analyzed. CKII peptides were attached to a cover-glass surface as described in *Material and Methods*.

To minimize unspecific binding, the peptide-coated glass surface was blocked with a 2 % BSA solution for 20 minutes, which was done for every CaM/CKII photounbinding experiment as well. A certain amount of labeled CaM attached to the glass surface unspecifically even though BSA blocking was done. The amount of unspecific CaM-

A647 rebinding in the presence of crosslinker, peptide, and CaM-A488 was determined as follows:

Before exposing CaM-A647 to the glass surface the binding site of CaM was blocked with a CKII(290-312)* peptide without a Cystein residue at the N-terminal end. Thus, this peptide could not bind to free SM(PEG)8 crosslinkers on the cover-glass surface. CKII(290-312)* peptide incubation (20 mM) for several hours ensured that all CaM binding sites are saturated. The CaM concentration was equal to the concentrations that were used at all other photounbinding experiments (details see *Material and Methods*). After CKII coating, the surface was incubated with CaM-A488 and a square was bleached onto the coated surface. Unspecific rebinding was measured on the CaM-A488/CKII coated surface to obtain unspecific rebinding levels as would occur for the photounbinding study. No CaM-A647 rebinding pattern was observed in the bleached square, which verifies that CaM-A647/CKII(290-312)* peptide attached to the surface unspecifically.

Fig. 22 illustrates the assay that was used to detect unspecific binding.

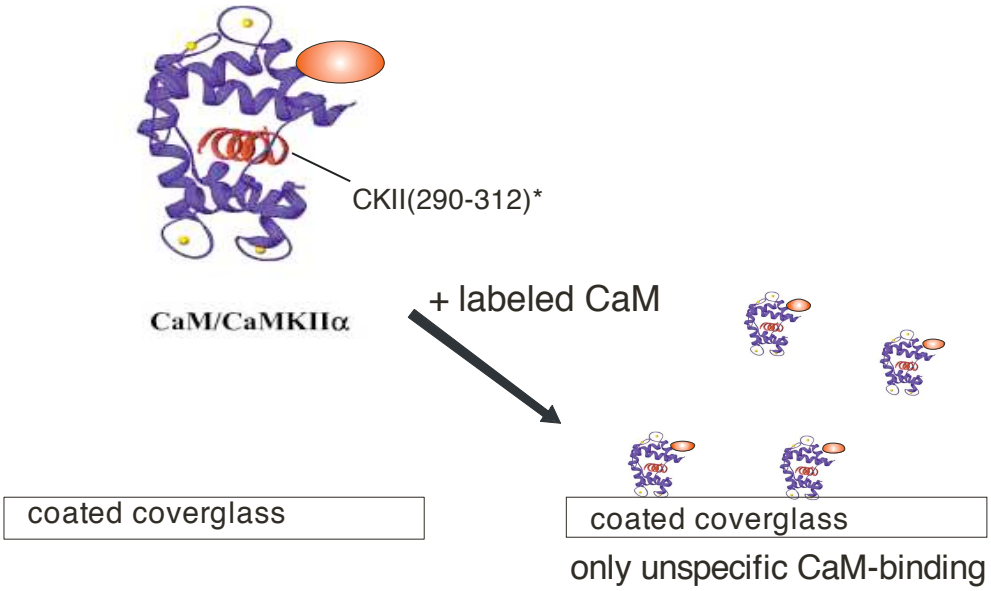
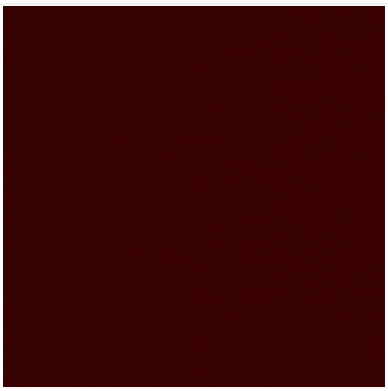


Fig. 22: Assay (unspecific binding); the binding site of CaM was blocked with a high affinity peptide (CKII(290-312) - see Material and Methods) which cannot attach to free crosslinker on the glass surface (there is no C- or N-terminal Cys residue) resulting in labeled CaM/CKII(290-312)* which can only attach unspecifically to the coated cover-glass. Based on this assay the amount of unspecific CaM-A647 rebinding was determined.*

Due to the blocked binding site of CaM, the resulting A647 fluorescence on the glass surface reflects the amount of unspecific rebinding. For quantification of unspecific

binding we repeated the experiment described above with non-blocked CaM and compared the respective average fluorescence intensities on the glass surface (fig. 23).



Negative control: CaM-A647/CKII(290-312)* rebinding on the CaM-A488/CKII surface
(no rebinding pattern observable – only unspecific binding)

Imaging parameters were the same for the negative and positive control:
488nm laser int. 0.3%;
633nm laser int. 5.0%;
Objective: Plan-Apochromat 63x/1.4 Oil DIC M27
Filters: Ch1 LP 505; Ch2 LP 650
Pinhole: 1000 µm

Positive control:
CaM-A647 incubation on CKII coated cover-glass

Imaging parameters:
488nm laser int. 0.3%;
633nm laser int. 5.0%;
Objective: Plan-Apochromat 63x/1.4 Oil DIC M27
Filters: Ch1 LP 505; Ch2 LP 650
Pinhole: 1000 µm

Fig. 23: images of positive and negative control to determine the amount of unspecific rebinding

The fraction of unspecific binding of labeled CaM was determined using the software ImageJ to approximately 9 %. This was considered for all related photounbinding experiments.
The results were obtained as following:

Negative control:
Mean Intensity (CaM-A647/CKII(290-312)* fluorescence) = 380 (gray value)

A positive control measurement was done using CaM-A647, without having its binding site blocked, to incubate the CKII (290-312) coated glass surface (fig. 23).
Mean Intensity = CaM A647 – control = 4094 (gray value)

Amount of unspecific rebinding:

$$380 * \frac{100}{4094} \sim 9\%$$

3.8. Photounbinding of fluorescent phalloidin in cells

Previous work has already shown that photounbinding affects antibody-antigen interactions (Heinze et al. 2009) when Alexa dyes, fluorescein or GFP is used as a fluorescent label.

The results presented in this study as well as in previous work (Akaaboune et al. 2002; Heinze et al. 2009) have shown that photounbinding occurs for various high affinity binding systems.

To further support the hypothesis that photounbinding occurs for various binding systems, even in the cellular environment, photounbinding of labeled phalloidin from F-actin was studied in fixed human fibrosarcoma cells.

Actin filaments of AAV-HT1080 cells were stained with a labeled phallotoxin. Phalloidin tightly binds actin subunits – $K_d = 3.6x10^{-8}$ M - (Faulstich et al. 1977) and stabilizes actin filaments (Barden et al. 1987). The cells were cultivated on chambered cover-glasses for two days at 37 °C and 5 % CO_2. After washing the chambered cover-glasses with prewarmed PBS, the AAV-HT1080 cells were stained with phalloidin Alexa 488 according to the protocol described in the *Material & Methods* section. To reduce background, the cells were preincubated in a PBS 1 % BSA solution for 30 minutes. Fig. 24 shows a phalloidin A488 stained AAV-HT1080 cell.

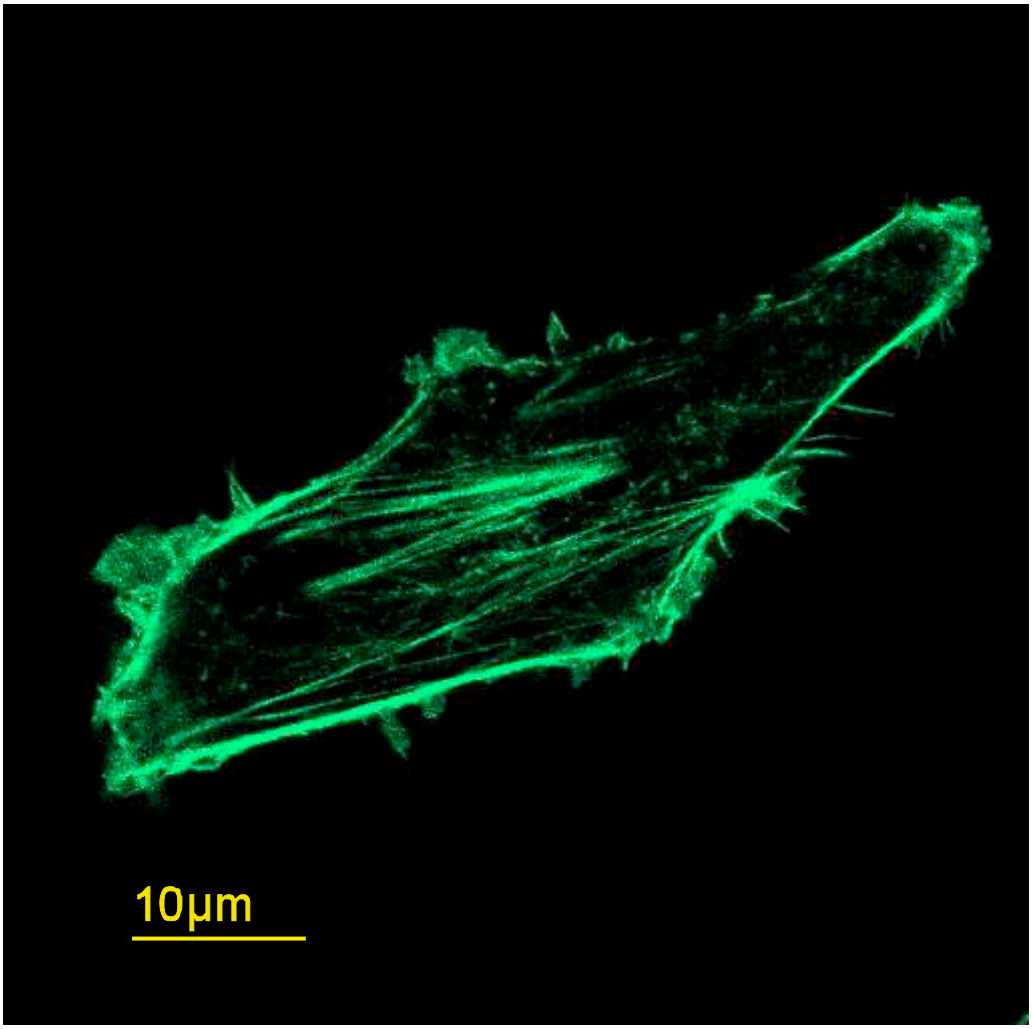


Fig. 24: Actin staining of an AAV-HT1080 human fibrosarcoma cell

A phalloidin concentration (methanolic stock [200 units/ml] was diluted 1:200 in PBS 1% BSA) was chosen that is sufficient to saturate nearly all free binding sites and an unbinding assay was performed on the stained cells with one and two photon excitation.

Actin filaments were bleached with different laser intensities (1PE – 488 nm, 20 μW-370 μW and 2PE – 800 nm, 14 mW – 25 mW) and incubated with phalloidin-A647 directly after bleaching to visualize binding sites that were available after photounbinding. Clear rebinding patterns were seen following phalloidin-A647 incubation, showing that labeled phalloidin can be unbound from actin filaments by light excitation (fig. 25).

Photounbinding assays (1PE) on actin filaments are shown in fig. 25 and fig. 26. Prior to bleaching, the chambered cover-glasses were filled with PBS (1 ml per chamber) to dilute photounbound phalloidin and avoid its rebinding (for details see *Material & Methods* section).

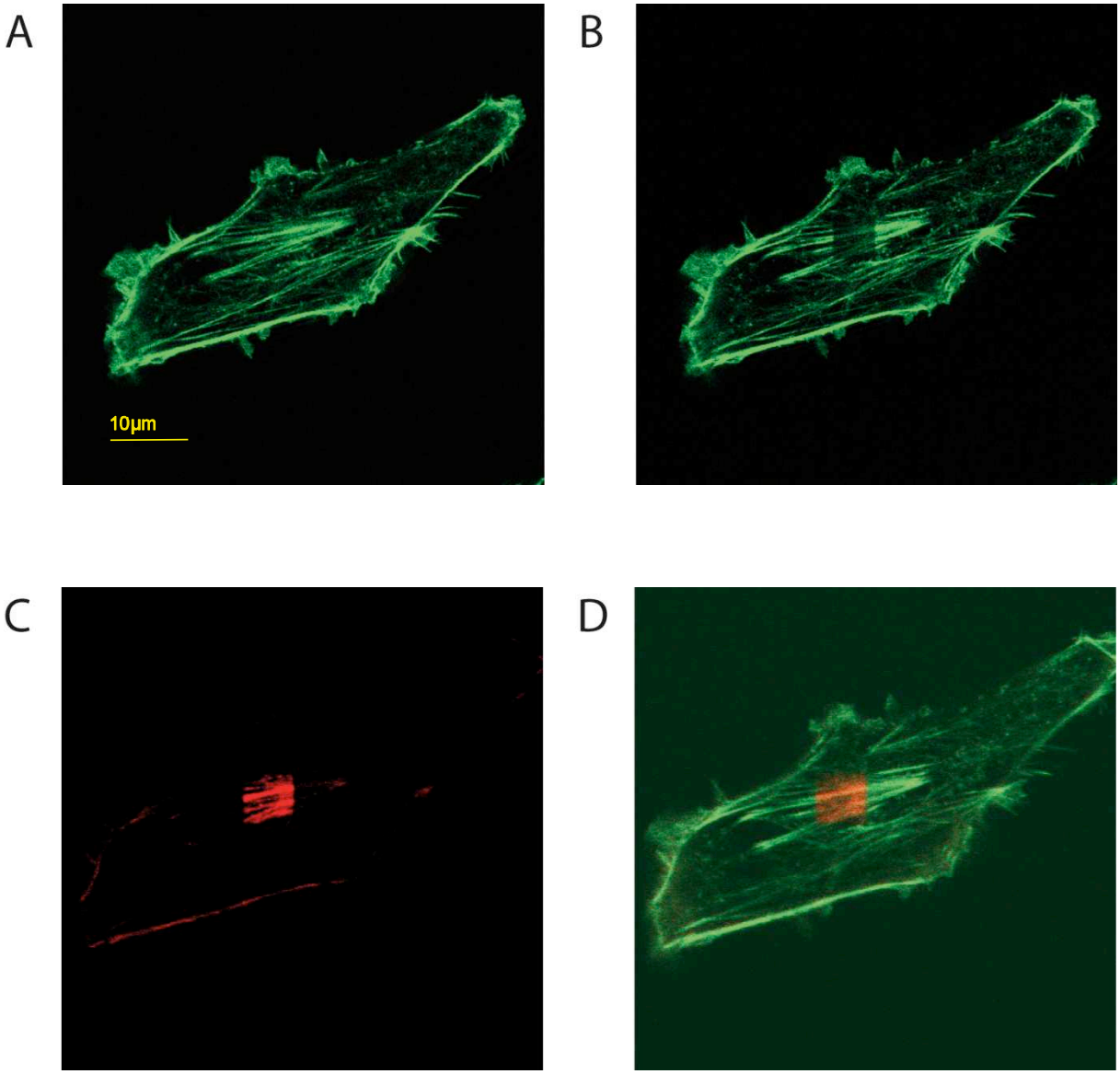


Fig. 25: Photounbinding assay on labeled actin filaments; (A) actin staining (phalloidin A488), (B) a small square was bleached onto actin filaments (one photon excitation, laser intensity: 23 µW; bleaching was done with 4 Iterations), (C) rebinding pattern of phalloidin A647, (D) overlay of green and red channel.

Light induced unbinding of phalloidin-A488 occurred at all tested laser intensities (1PE, 488 nm; 20, 90, 185 & 370 μW ; 2PE, 800 nm; 14, 20, 24, 25 & 32 mW). Even very low intensities like 20 μW (1PE) were sufficient to intracellularly photounbind phalloidin from actin filaments (fig. 26).

The tested intensities are commonly used in confocal microscopy. It has been shown in previous work (Heinze et al. 2009) that laser intensities that are typically used for imaging can already induce photounbinding of antibody-antigen interactions.

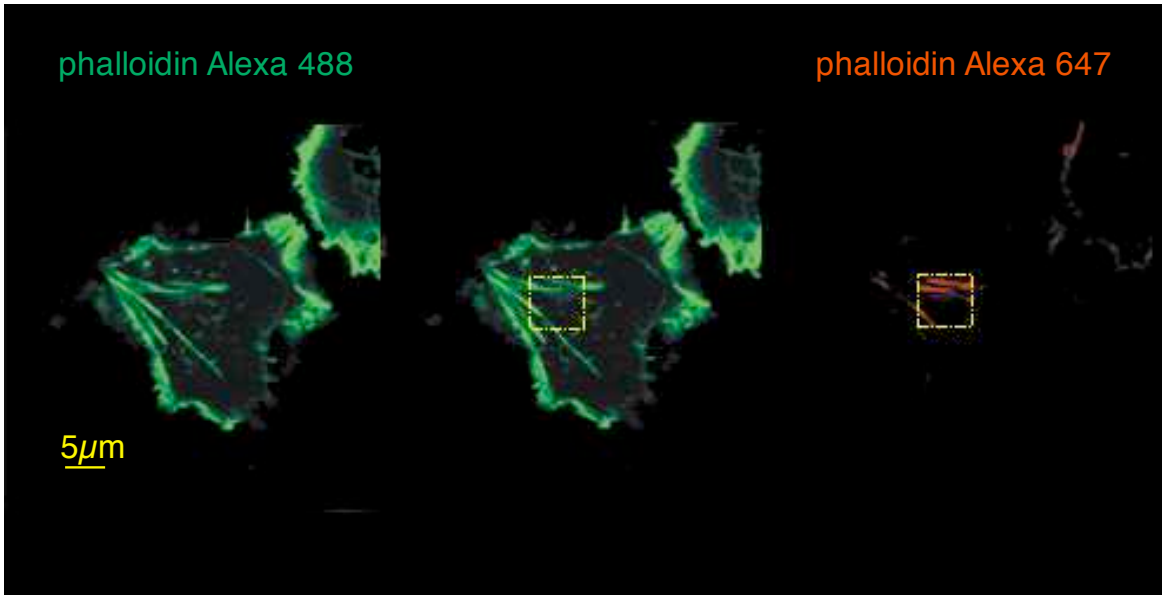
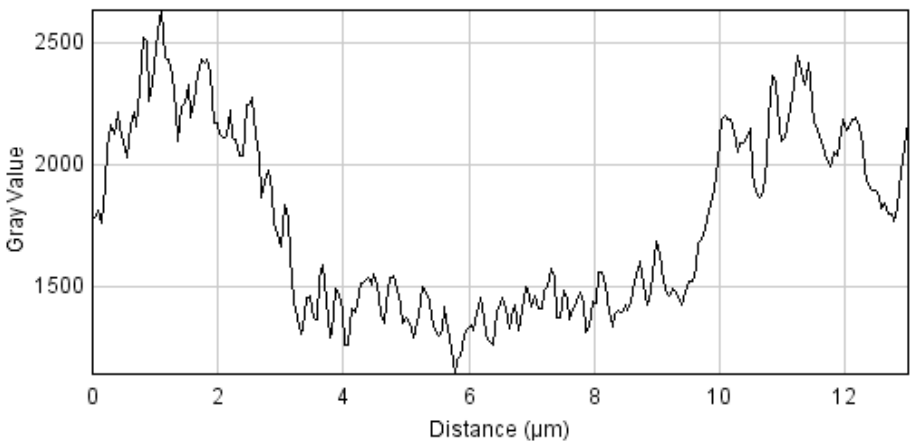


Fig. 26: photounbinding of labeled phalloidin from actin filaments; (bleaching: 1PE, 488 nm, 20 µW)

The surface plots in fig. 27 visualize photounbinding after 1PE along actin filaments. The decrease of the phalloidin-A488 fluorescence correlates with an increase of the phalloidin-A647 fluorescence, meaning that specific rebinding occurred in the bleached region.

A clear increase of the phalloidin-A647 fluorescence compared to the non-bleached neighboring area was observed.

A



B

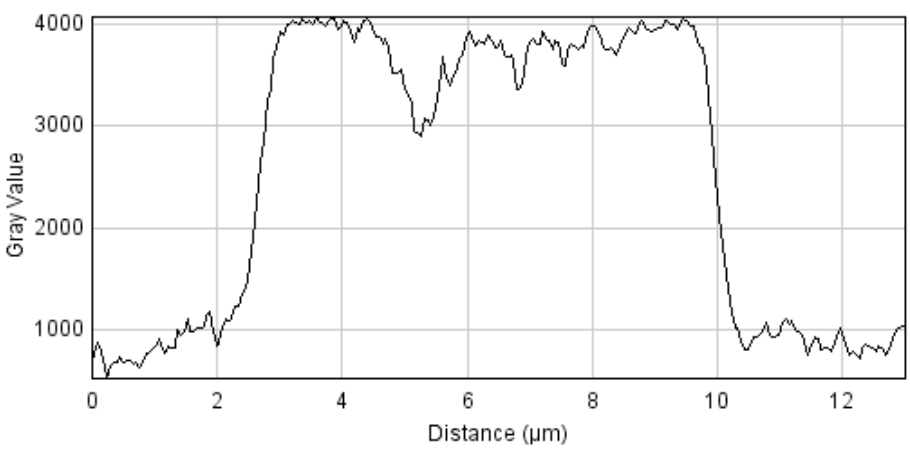


Figure 27: surface plots of actin filaments; phalloidin A488 fluorescence (A), phalloidin A647 fluorescence (B). The bleached area starts at approximately 2.5 µm and stops at 10 µm (1 PE, 488 nm, 185 µW).

To test if phallotoxins can be unbound by two photon excitation as well, the experiment was repeated and bleaching was done by two photon excitation with 800 nm.

A clear rebinding pattern was seen, demonstrating that labeled phalloidin can be dissociated from actin filaments by two photon excitation [fig. 28 (B)] as well.

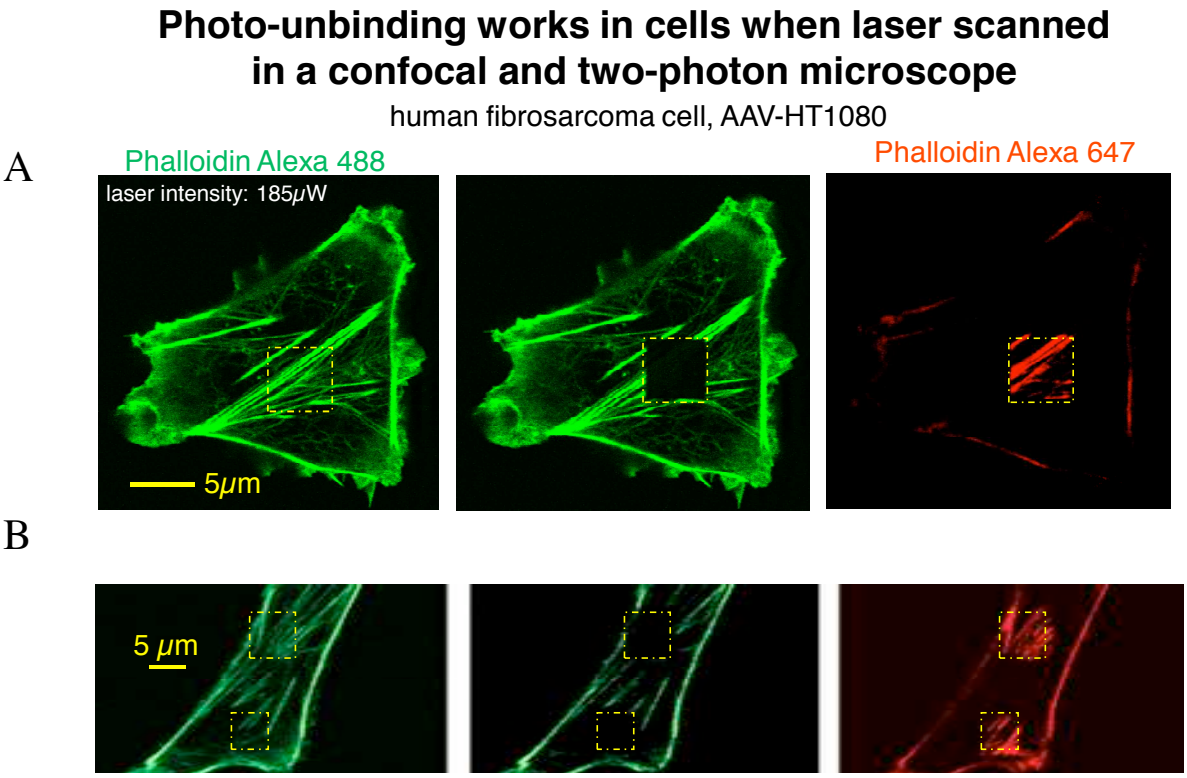


Fig.28: photounbinding of labeled phalloidin by 1PE and 2PE; (A) 1PE, bleaching: 185 µW; (B) 2PE, bleaching: square (bottom) – 24 mW, 8 iterations; square (top) – 25 mW, 1 iteration

Fig. 29 shows a human fibrosarcoma cell at which photounbinding was done by 2PE with laser intensities of 14, 20 and 24 mW. All applied bleaching intensities induced significant photounbinding.

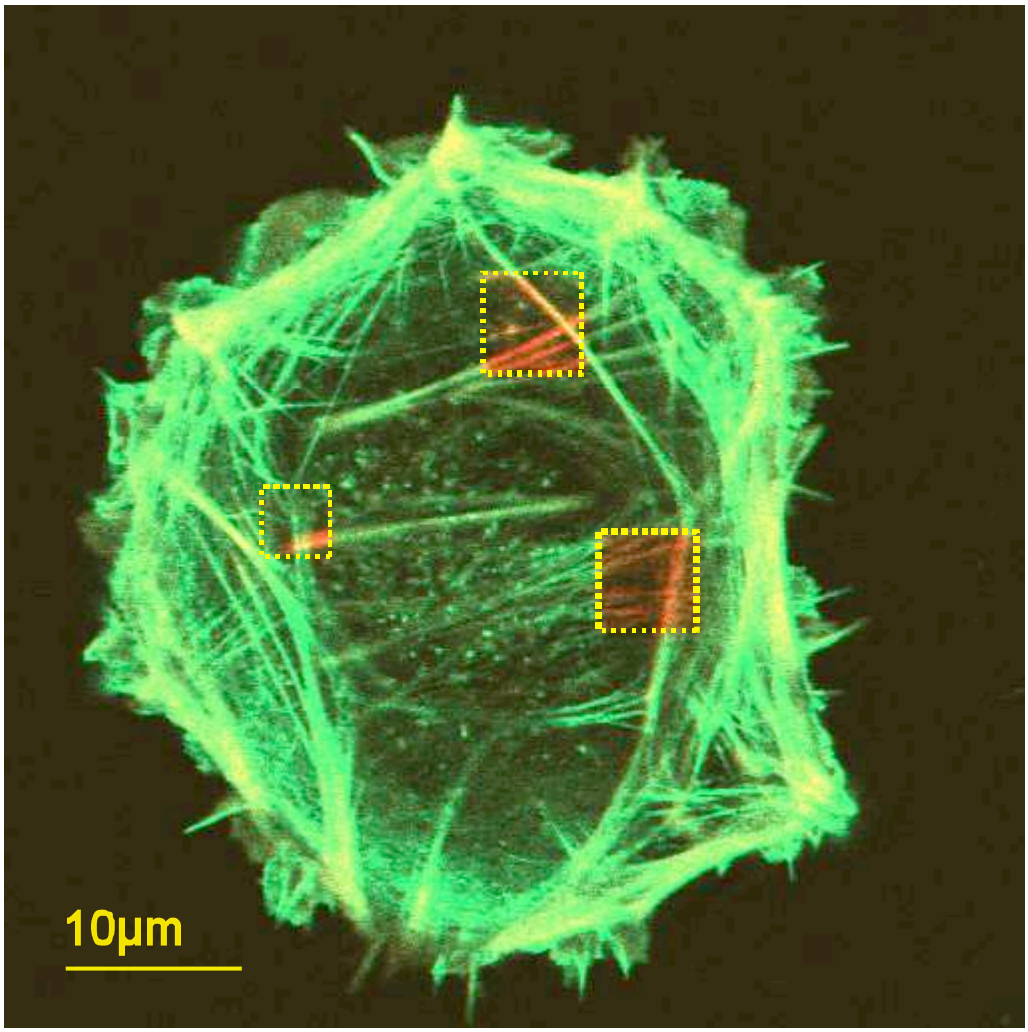


Fig. 29: photounbinding assay on a human fibrosarcoma cell, three squares were bleached applying 2PE (800 nm), square-left: 14 mW; square-top mid: 20 mW; square-right: 24 mW

The obtained results show that labeled phalloidin can be dissociated from actin filaments by light excitation. However, photounbinding cannot be observed for unlabeled phalloidin. For the respective experiment, actin filaments were incubated with unlabeled and labeled phalloidin at a ratio of 4:1 and excited with laser light (488 nm with 1PE and 800 nm with 2PE). A small portion of labeled phalloidin was necessary to 'visualize' the actin filaments in the microscope and set the focus for bleaching (for details see *photounbinding assay actin/phalloidin*). If unlabeled phalloidin could be unbound as well, a clear rebinding pattern, as obtained for exclusively labeled phalloidin, should be seen after phalloidin-A647 reincubation. If only a small fraction of labeled phallotoxin is dissociated from the actin filaments by light, little or no rebinding pattern would be detectable. After bleaching and reincubation with phalloidin-A647, the cells were imaged and analyzed.

Fig. 30 shows surface plots of actin filaments after bleaching and reincubation.

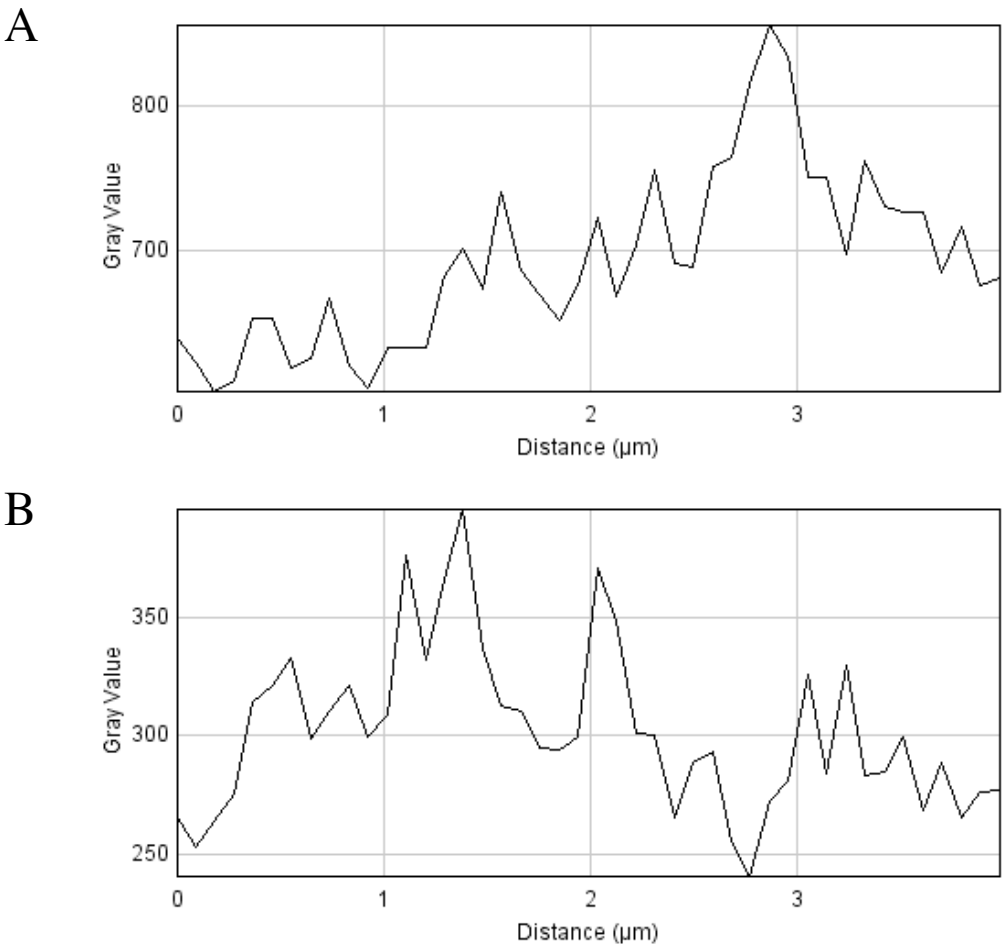


Fig. 30: surface plots of actin filaments (primary staining: unlabeled and labeled phalloidin at a ratio of 4:1) after bleaching and reincubation (the bleached area ranges from 0 to 2.5 µm); (A) phalloidin-A488 fluorescence; (B) phalloidin-A647 fluorescence

Only a slight increase of the phalloidin-A647 fluorescence in the bleached area was detectable. Compared to the non-bleached neighboring area, an increase of 2.2 % in the phalloidin-A647 fluorescence was observable in the bleached region. The control measurement (for which only labeled phalloidin was used) showed a 75 % increase of the phalloidin-A647 fluorescence (fig. 31). For the first incubation step a phalloidin fraction of 20 % was labeled - this could be the maximal amount of unbound phalloidin. The amount of detectable rebound phalloidin is below the fluorescently labeled fraction, most probably due to the fact that the majority of labeled phalloidin gets bleached and not unbound by laser excitation (this was also observed with labeled CaM – see 3.1 and 3.2). However, background staining could also bias quantitative analysis.

The small rebinding values (only 2.2 % increase of the CaM-A647 fluorescence compared to the non bleached actin filaments) indicate that unlabeled phallotoxins cannot be unbound by laser excitation. Otherwise enhanced or even similar rebinding levels as

obtained in the control measurement (75% increase of the phalloidin-A647 fluorescence compared to the non bleached area) would occur.

Surface plots of actin filaments of the control measurement show a clear increase of the A647 fluorescence in the bleached region (fig. 31), which is absent when unlabeled phalloidin was used in excess.

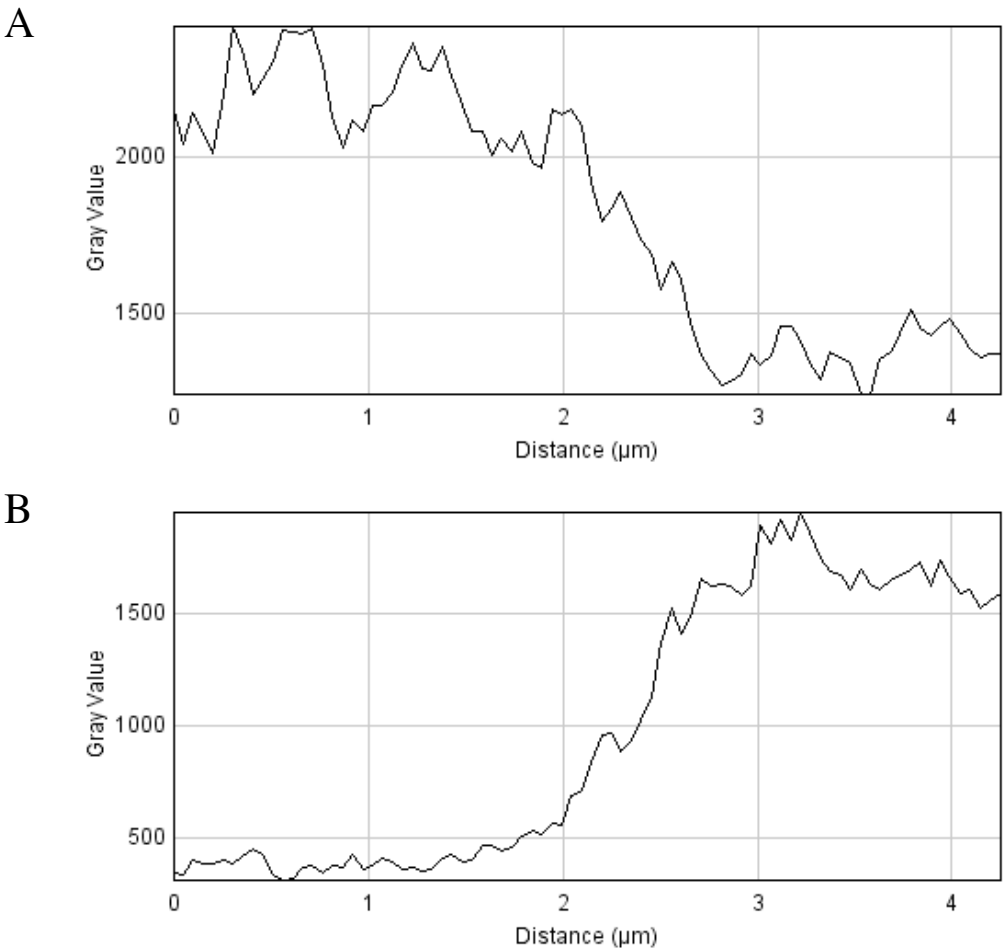


Fig. 31: surface plots of actin filaments after bleaching and reincubation with phalloidin-A647 (the bleached area ranges from ~2.5 µm to 4.3 µm; 1PE, 185 µW); (A) phalloidin-A488 fluorescence, (B) phalloidin-A647 fluorescence

4. Discussion and Outlook

Photounbinding has been shown to occur for various common binding systems such as antibody-antigen (Heinze et al. 2009)-, protein-peptide (this work)-, as well as toxin-target interactions (Aakaboune et al. 2002, this work). It occurs in solution (Heinze et al. 2009, this work), in cell culture (this work) as well as *in vivo* (Aakaboune. et al 2002). In this thesis work it was demonstrated that labeled CaM in solution and phalloidin in cells were dissociated by one and two photon excitation, while leaving their binding partners intact. The unbinding efficiencies measured in this and previous work were substantial and ranged between a maximum of 20% (this work) and 85 % (Heinze et al. 2009) dependent on the molecular system under investigation and the excitation mode.

Photounbinding was visualized by rebinding the same, but differently labeled, binding partners. We observed that rebinding occurred only in the previously illuminated (photobleached) areas. In case of Calmodulin-(CKII) peptide binding in solution we have developed an assay and a respective analysis routine which allowed indirect quantification of the amount of unbound protein by considering the fraction of rebound protein after laser illumination. The (CKII) peptides that were synthesized for this study differed in their length by one amino acid each, resulting in different dissociation constants for Calmodulin. Thus, this system is ideally suited to study the dependence of photounbinding on the Kd without changing the molecular system under study.

Our experiments revealed that photounbinding

a) is reversible
b) requires a fluorescent label
c) occurs for various fluorescent labels
d) occurs in both solution and in cells
e) occurs after both one- and two-photon excitation;
f) is strongly dependent on the initial binding constant of the molecular system as it occurs more likely with increased binding affinity;
g) is linked to photobleaching and has comparable laser thresholds
h) does not break covalent bonds

We would like to discuss details in the following:

a) Photounbinding is reversible and occurs both in solution an in cells

The assay based on unbinding and rebinding of proteins in solution we have developed, clearly shows that laser light can cause the reversible dissociation of proteins from their binding partner. In an intracellular environment (human fibrosarcoma cell line) we showed that labeled phalloidin can be selectively unbound from actin filaments by laser scanning and rebound with a different color without disrupting the actin filaments. The dissociation constant of phalloidin-A488 or -A647 has not be determined so far but it is presumably in the range of $3.6x10^{-8}$ M, which is the K_d of unlabeled phalloidin (Faulstich et al. 1977). This relatively high affinity binding was dissociated by one and two photon excitation.

b) Photounbinding depends on the initial binding constant of the molecular system

It has been demonstrated that the five different CKII peptides selected (CKII(290, 291, 292, 293, 294-312)) show different rebinding levels to CaM-A647. The amount of rebound Calmodulin differed by a factor of ~9 (mean values of three measurements) between CKII(290-312, highest binding affinity) and CKII(294-312, lowest binding affinity) when bleaching was done by one photon excitation. Although the Calmodulin-CKII(290-312) interaction has a Kd of $3x10^{-13}$, a high amount (up to ~20%) of labeled Calmodulin was dissociated by light excitation. With increasing dissociation constants of the CKII peptide/Calmodulin interaction, the photounbinding effect is decreasing. We conclude that photounbinding is more likely to occur if the dissociation constant is low.

The obtained results were corrected mathematically to minimize measurement errors that would arise particularly for the peptides with a higher K_d (higher off-rates) where CaM may diffuse off the CKII peptide coated surface before the experiment has been finished (illumination, rinsing, re-incubation) and thus may be lost for the final imaging step.

The CaM-CKII off rates, which had been measured by Waxham and colleagues at room temperature, would show a 2-4 fold decrease when the temperature is lowered to 4°C (Waxham et al. 1998). To avoid overestimation in differences of photounbinding between the various CKII peptides, the decrease of the off rates was assumed to be only two-fold (the CaM/CKII peptide coated surface was overlaid with buffer at a temperature of 4°C).

Finally, rebinding values of CaM-A647 to the CKII(294-312) peptide were shifted after correction, but the overall tendency of the observed rebinding intensities to different CKII

peptides did not change significantly following data correction, because of the other peptides showing small off rates.

c) Photounbinding is linked to photobleaching

In our experiments the window of laser power where photobleaching occurs, but photounbinding does not was non existant or rather small. This raises the question of whether photobleaching and photounbinding occur independently or just back-to-back (bleaching first, then unbinding). By addition of the chemical stabilizer ascorbic acid, it has been shown that photobleaching after two-photon excitation can be reduced. The lifetime of a fluorescent dye is extended as ascorbic acid helps to prevent the two-photon bleaching pathway by its reducing properties (Dittrich et al. 2001).

Dependent on the mechanism underlying photounbinding we have tested the two possible hypothesis after adding ascorbic acid,: 1. Unbinding/rebinding stays equal, which would imply that photounbinding occurs independently from bleaching; 2. Unbinding/rebinding is decreased as the fluorescence is stabilized indicating that photounbinding occurs subsequently to photobleaching and thus is directly linked to it. We have observed the second case, which suggests that artifacts in fluorescent techniques due to photounbinding could be entirely prevented when photobleaching is avoided.

d) Photounbinding requires a fluorescent label

It seems that the presence of a label is a requirement for photounbinding, as it was shown that unlabeled CaM in solution and phalloidin in cells failed to be dissociated from their targets by light excitation (1PE 488nm and 2PE 800nm). If the label has to be fluorescent or if absorption would be sufficient to induce dissociation would be another important hint towards the mechanism of photounbinding. This question is under current investigation; experiments where the fluorescent label on CaM is replaced by a so-called quencher dye with a high extinction coefficient, but negligible fluorescence quantum efficiency are planned.

If an absorbing but non fluorescent label would be sufficient for dissociation, it could eventually be possible that photounbinding occurs with unlabeled proteins as well as when the right excitation wavelength is used. When excitation is performed within the lower UV wavelength range, the protein itself absorbs. Aromatic amino acids like Tyrosine, Tryptophan and Phenylalanine absorb in the UV range and therefore proteins show significant absorption at 280 nm. This absorption property is commonly used for the

determination of protein concentrations for example. One could speculate that strong UV excitation (at 280nm for example) could eventually crack protein interactions.

e) Photounbinding occurs for various fluorescent labels

Another important finding is that photounbinding is not limited to a specific fluorophore or a certain excitation-wavelength. This was confirmed by photounbinding of Calmodulin which was attached either to A488 or to A647. Both dye conjugated proteins have been unbound by light excitation, showing that photounbinding is not restricted to a specific dye or excitation wavelength. It is not known yet to which extent different dyes influence photounbinding. For future work it would be interesting to find out if or how different labels influence photounbinding properties.

f) Photounbinding does not break covalent bonds

As shown in this work, labeled CaM could not be unbound from CKII peptides after formaldehyde fixation. Formaldehyde fixation results in strong covalent crosslinking of Calmodulin and CKII peptides. This linkage could neither be "broken up" by one nor two photon excitation. It seems that a covalent linkage is too stable to allow light induced unbinding. This finding is supported by a different experiment with GFP actin fusion proteins in cells, where GFP failed to be dissociated when GFP-actin fusion proteins were laser illuminated.

In conclusion, our finding suggest that ─ besides the well known risk of photobleaching ─ dissociation constants of various peptide-protein interactions are indeed affected by photounbinding. Notably the laser intensity threshold for photobleaching was always found to be the same or similar as for the observed photounbinding effect, so that the laser intensity window where photobleaching occurs, but no photounbinding can be observed is rather small or even not existent.

It was shown that laser intensities of 5mW or higher (1PE, 489nm, 2 iterations) were sufficient to almost completely bleach the labeled CaM and photounbind up to 20 % of the originally bound CaM from its ligand. Notably, already ~70 μW laser power (1PE, 489nm, 1 iteration), was sufficient to unbind Calmodulin. The phalloidin-A488/actin binding was dissociated even when exposed with laser intensities as low as 20μW (1PE, 488nm).

Particularly in FRAP (Fluorescence Recovery after Photobleaching) or FLIP (Fluorescence Loss in Photobleaching) experiments photounbinding could be misinterpreted as bleaching and bias the obtained results. With FRAP for example, photounbound proteins within the 'bleached' area could recruit binding partners from the outside 'recovering' proteins and change the binding equilibrium and mobility constants of those significantly and unintentionally. Thus, the risk of photounbinding should be taken into account when performing experiments based on fluorescence techniques involving photobleaching.

Even if the basic mechanism of photounbinding is still largely unknown, this work together with previous studies (Heinze et al. 2009; Akaaboune et al. 2002) has lead to further understandings of photounbinding and helps in various fields of bio-imaging and biomedical research.

5. References

Akaaboune M., Grady M.R., Turney S., Sanes J.R. & Lichtman J.W. Neurotransmitter Receptor Dynamics Sudied In Vivo by Reversible Photounbinding of Fluorescent Ligands. *Neuron* **34**, 865-876 (2002).

Barden J.A., Miki M., Hambly B.D. & Dos Remedios C.G. Localisation of the Phalloidin and Nucleotide-Binding Sites on Actin. *Eur. J. Biochem.* **162**, 583-588 (1987)

Baumgarth B., Bartels F.W., Anselmetti D., Becker A. & Ros R. Detailed Studies of the Binding Mechanism of the Sinorhizobium Meliloti Transcriptional Activator ExpG to DNA. *Microbiology* **151**, 259-268 (2005).

Bulina M.E., Chudakov D.M., Britanova O.V., Yanushevich Y.G., Staroverov D.B., Chepurnykh T.V., Merzlyak E.M., Shkrob M.A., Lukyanov S. & Lukyanov K.A. A Genetically Encoded Photosensitizer. *Nat. Biotechnol.* **24(1)**, 95-99 (2006)

Chen T.S., Zeng S.Q., Luo Q.M., Zhang Z.H. & Zhuo W. High-order Photobleaching of Green Fluorescent Protein inside Live Cells in Two-photon Excitation Microscopy. *Biochem. Biophys. Res. Commun.* **291**, 1272-1275 (2002)

Dittrich P.S. & Schwille P. Photobleaching and Stabilization of Fluorophores Used for Single-Molecule Analysis with One- and Two-Photon Excitation. *Appl. Phys.* B **73**, 829–837 (2001)

Dobrucki J. W., Feret D. & Noatynsky A. Scattering of Exciting Light by Live Cells in Fluorescence Confocal Imaging: Phototoxic Effects and Relevance for FRAP Studies. *Biophysical J.* **93**, 1778-1786 (2007)

Faulstich H, Schäfer A.J. & Weckauf M. The Dissociation of the Phalloidin-Actin Complex. *Hoppe Seylers Z Physiol Chem.* (1977)

Heinze K.G., Costantino S., De Koninck P. & Wiseman P.W. Beyond Photobleaching, Laser Illumination Unbinds Fluorescent Proteins. *J. Phys. Chem. B.* **113**, 5225-5233 (2009)

Heinze K.G., Rarbach M., Jahnz M. & Schwille P. Two-Photon Fluorescence Coincidence Analysis: Rapid Measurements of Enzyme Kinetics. *Biophysical J.* **83**, 1671-1681 (2002)

Hoebe R.A., Van Der Voort H.T.M., Stap J., Van Noorden C.J.F. & Manders E.M.M. Qantitative Determination of the Reduction of Phototoxicity and Photobleaching by Controlled Light Exposure Microscopy. *J. Microsc.* **231** (Pt 1), 9-20 (2008)

Hudmon A. & Schulman H. Structure-Function of the Multifunctional Ca2+/Calmodulin-Dependent Kinase II. *Biochem. J.* **364**, 593-611 (2002)

Kim S.A., Heinze K.G., Bacia K., Waxham M.N. & Schwille P. Two-Photon Cross-Correlation Analysis of Intracellular Reactions with Variable Stoichiometry. *Biophysical J.* **88**, 4319-4336 (2005)

Kim S.A., Heinze K.G. & Schwille P. Fluorescence Correlation Spectroscopy in Living Cells. *Nat. Meth.* **4**, 963-973 (2007)

König, K., Riemann, I. & Fritzsche W. Nanodissection of Human Chromosomes with Near-Infrared Femtosecond Laser Pulses. *Opt. Lett.* **26**, 819-821 (2001)

Kudryashov D.S., Sawaya M.R., Adisetiyo H., Norcross T., György H., Reisler E. & Yeated T.O. The Crystal Structure of a Cross-Linked Actin Dimer Suggests a Detailed Molecular Interface in F-Actin. *PNAS* **102**, 13105-13110 (2005)

Meyer T., Hanson L., Stryer L. & Schulman H. Calmodulin Trapping by Calcium-Calmodulin-Dependent Protein Kinase. *Science* **256**, 1199-1202 (1992)

Panchuk-Voloshina N., Haugland R.P., Bishop-Stewart J., Bhalgat M.K., Millard P.J., Mao F., Leung W. & Haugland R.P. Alexa Dyes, a Series of New Fluorescent Dyes that Yield Exceptionally Bright, Photostable Conjugates. *The Journal of Histochemistry & Cytochemistry* **47**, 1179-1188 (1999)

Pellicena P. & Kuriyan J. Protein–Protein Interactions in the Allosteric Regulation of Protein Kinases. *Current Opinion in Structural Biology* **16**, 702-709 (2006)

Remington S.J. Fluorescent Proteins: Maturation, Photochemistry and Photophysics. *Curr. Opinion in Struct. Biol.* **16**, 714-721 (2006)

Song L., Hennink E.J., Young T.I. & Tanke H.J. Photobleaching Kinetics of Fluorescein in Quantitative Fluorescence Microscopy. *Biophysical J.* **68**, 2588-2600 (1995)

Swulius M.T. & Waxham M.N. Ca^{2+}/Calmodulin-dependent Protein Kinases. *Cell. Mol. Life Sci.* **65**, 2637-2657 (2008)

Vetter S.W. & Leclerc E. Novel Aspects of Calmodulin Target Recognition and Activation. *Eur. J. Biochem.* **270**, 404-414 (2003)

Waxham N. M., Tsai A. & Putkey A. Mechansim for Calmodulin (CaM) Trapping by CaM-kinase II Defined by a Family of CaM-binding Peptides. *J. Biol. Chem.* ***273****, 17579-17584* (1998)

White J. & Stelzer E. Photobleaching GFP Reveals Protein Dynamics inside Living Cells. *Trends in Cell Biol.* **9(2)**, 61-62 (1999)

6. Abbreviations

Å	Ångstrom
A488, -546, -568, -647	Alexa Fluor 488, -546, -568, -647
BSA	bovine Serum albumin
$CaCl_2$	calcium chloride
CALI	chromophore-assisted light inactivation
CaM	calmodulin
CKII	calcium/calmodulin dependent protein kinase II
CO_2	carbon dioxide
Cys	cysteine
ddH_2O	double distilled water
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FCS	fetal calf serum
FRAP	Fluorescence Recovery after Photobleaching
FLIP	Fluorescence Loss in Photobleaching
GFP	green fluorescent protein
HCL	hydrochloric acid
HNO_3	nitric acid
HPLC	high performance liquid chromatography
KCL	potassium chloride
K_d	dissociation constant
kDa	kilo Dalton
k_{off}	off rate
k_{on}	on rate
LSM	laser scanning microscope
μ	micro
ml	millilitre
mM	millimolar
MOPS	3-morpholinopropanesulfonic acid
mW	milliwatt

N	nitrogen
nm	nanometer
PBS	phosphate-buffered saline
PFA	paraformaldehyde
ROS	reactive oxygen species
RT	room temperature
$SM(PEG)_8$	NHS-PEG_n-Maleimide crosslinker (Succinimidyl-([N-maleimidopropionamido]-#ethylenglycol) ester
Thr	threonine
UV	ultraviolet

1PE	one-photon-excitation
2PE	two photon excitation

Summary

Recent studies have shown that labeled binding partners can be reversibly dissociated by laser light if at least one binding partner carries a fluorescent label. It was observed that labeled antibody-antigen (Heinze et al. 2009) and toxin-target (Akaaboune et al. 2002) interactions can be unbound by light. This phenomenon, referred to as photounbinding in the literature is further characterized in this work with an emphasis on the molecular mechanism of light induced unbinding of protein-peptide interactions. Unbinding of labeled Calmodulin (CaM) from a set of CaM-binding peptides with different affinities to CaM is analyzed and quantified. We show that protein-peptide interactions can also be dissociated by one- and two-photon excitation whereas photounbinding of labeled CaM from Calcium/Calmodulin dependent Protein Kinase II (CKII)-peptides is more likely to occur if the dissociation constant is low. Furthermore, it is shown that light induced dissociation of labeled proteins is linked to a process referred to as photobleaching, thus becoming relevant shortly *after* or *with* the occurrence of photobleaching. These experiments were performed in solution which requires immobilization of peptides onto a glass-bottom petri dish. After CaM-incubation on a peptide coated cover-glass one- and two photon excitation in defined regions with a Laser Scanning Microscope of a defined protein peptide layer were performed. The basic idea is that bleached CaM-A488 remains bound to the peptide coated surface whereas light induced dissociation results in a vacant binding site. Finally, photounbinding was visualized and analyzed by subsequent rebinding of differently labeled CaM.

Additionally, experiments in cell culture demonstrate that labeled toxin-target interactions are dissociated by one- and two-photon excitation as well. Thereby fluorescently labeled phalloidin was selectively dissociated from actin filaments within cells. Again, vacant binding sites, following light induced dissociation, were visualized by subsequent rebinding of a differently labeled binding partner.

This work shows that photounbinding already occurs at low laser intensities that cause only slight bleaching and therefore is relevant for several quantitative fluorescent studies, especially for those which use photobleaching as a tool to analyze protein mobility or interactions like for example Fluorescence Recovery after Photobleaching (FRAP).

Zusammenfassung

In kürzlich veröffentlichten Arbeiten wurde gezeigt, dass fluoreszenzmarkierte Bindungspartner durch Lichtanregung dissoziiert werden können (Akaaboune et al. 2002; Heinze et al. 2009). Dieses Phänomen, in der Literatur als „Photounbinding“ bezeichnet, wurde in dieser Arbeit weiter charakterisiert. Im Besonderen wurde auf den molekularen Mechanismus der Licht-induzierten Dissoziation von Protein/Peptid Bindungspaaren eingegangen. In dieser Studie wurde die Bindung von Calmodulin (CaM) zu CaM-bindenden Peptiden verschiedener Affinität, untersucht und die Ergebnisse quantitativ ausgewertet. Die durchgeführten Experimente haben gezeigt, dass Protein/Peptid Bindungen durch Lichtanregung dissoziiert werden können, falls wenigstens einer der Bindungspartner fluoreszenzmarkiert ist. Dabei steigt die Wahrscheinlichkeit für die lichtinduzierte Dissoziation mit wachsender Dissoziationskonstante des Calmodulin/Peptid. Desweiteren konnten die Experimente zeigen, dass „Photounbinding“ in Abhängigkeit vom Prozess des Photo-Bleichens auftritt, also kurz *nach* oder *mit* dem Eintreten von Photobleichen relevant wird. Für die Experimente wurden Peptide auf einem Deckglass immobilisiert, so dass nach Inkubation mit fluoreszenzmarkiertem CaM eine definierte Schicht Peptid-Protein an die Glasoberfläche binden und im Laser Scanning Mikroskop (LSM) mittels Ein-und Zwei-Photonen-Anregung bestrahlt werden konnte. Die Grundidee ist, dass ein durch Lichteinstrahlung ‚gebleichtes’ fluoreszenzmarkiertes CaM ─wenn auch unsichtbar in der Fluoreszenzmikroskopie─ die Bindungsstelle besetzt hält während ein Dissoziationsprozess (‚photounbinding’) eine vakante Bindungsstelle hervorbringen würde. Vakante Bindungsstellen konnten durch das Rückbinden eines andersfarbig fluoreszenzmarkierten CaM sichtbar gemacht und quantifiziert werden.

Weitere Experimente in Zellkultur konnten darlegen, dass „Photounbinding“ ebenfalls bei Toxin-Bindungen auftritt. Dabei wurde nachgewiesen, dass markiertes Phalloidin von Aktin Filamenten selektiv dissoziiert werden kann ohne die Aktin-Filamente zu zerstören. Freie Bindungsstellen wurden auch hier durch Rückbinden eines andersfarbigen Phalloidins visualisiert.

Da schon bei leichtem ‚Photobleichen’ auch ‚photounbinding’ beobachtet werden konnte sind die in dieser Arbeit gewonnenen Erkenntnisse für verschiedene quantitative Fluoreszenz-Analyseverfahren relevant, besonders dort, wo Photobleichen als Werkzeug

zur Untersuchung von Protein Mobilität oder Interaktion eingesetzt wird, wie z.B. für Anwendungen von Fluorescence Recovery after Photobleaching (FRAP).

Acknowledgements

First of all I would like to thank my group!

Thanks to my supervisor Dr. Katrin Heinze for her help, support and for giving me the opportunity to do my diploma project in her group!
Thanks to Kareem for the fun and the interesting scientific discussions!

Many thanks to Professor M. Neal Waxham (University of Texas Medical School at Housten) for scientific discussions and for providing Calmodulin.

I would also like to thank my friends I got to know at the Vienna Biocenter. Thanks for the great time here and for the interesting scientific exchange! I hope we can stay in touch although there may be some spatial distance between us.

Last but not least I would like to thank my family for their support and encouragement.

Klaus Gerald Neumüller
Teichstraße 22
4203 Altenberg
Tel. +43 650 8430041
e-mail: klaus_neumueller@gmx.at

Curriculum Vitae

Date and Place of Birth: 30/ 03/ 1983 in Linz, Austria
Nationality: Austrian

Education:

1994 – 2002	Grammar School in Linz, (Europagymnasium Linz-Auhof); main focus on science; A-levels 06/2002
10/2002 – 10/2003	civil service in the hospital AKH (= Allgemeines Krankenhaus), Linz
10/2003 – now	student of Biology at the University of Vienna since 02/2005: specialisation on Genetics and Microbiology in the second part of my academic studies

Work and Research experience:

04/2008 – 04/2009	Diploma Student at the IMP - Research Institute of Molecular Pathology, Vienna, in the group of Katrin Heinze project: towards the mechanism of protein-peptide photounbinding
04/2006 – 11/2007	Technical Assistant at the Institute of Molecular Biotechnology (IMBA) of the Austrian Academy of Sciences, in the group of Jürgen Knoblich project: genome wide transgenic RNAi screen