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Citation for the original published paper (version of record):

Wu, Y-W., Venkatachalapathy, M., Kamps, D., Weigel, S., Kumar, R. et al. (2017)
"Molecular Activity Painting": Switch-like, light-controlled perturbations inside living cells
Angewandte Chemie International Edition, 56(21): 5916-5920
<https://doi.org/10.1002/anie.201611432>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

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“Molecular Activity Painting”: Switch-like, light-controlled perturbations inside living cells

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Abstract: Acute subcellular protein targeting is a powerful tool to study biological networks. However, signaling at the plasma membrane is highly dynamic, making it difficult to study in space and time. In particular, sustained local control of molecular function is challenging due to lateral diffusion of plasma membrane targeted molecules. Here we present “Molecular Activity Painting” (MAP), a novel technology which combines photoactivatable chemically induced dimerization (pCID) with immobilized artificial receptors. The immobilization of artificial receptors by surface-immobilized antibodies blocks lateral diffusion, enabling rapid and stable “painting” of signaling molecules and their activity at the plasma membrane with micrometer precision. Using this method, we show that painting of the RhoA-myosin activator GEF-H1 induces patterned acto-myosin contraction inside living cells.

Keywords: Optochemical biology • Photochemically induced dimerization • Rho GTPases • Signal transduction • Immobilization

Cellular membranes can serve as platforms for signaling molecules. The plasma membrane of eukaryotic cells is an especially important platform that integrates external stimuli and internal states to control cellular processes^[1]. Chemical and optogenetic tools are powerful approaches to acutely modulate protein function by protein translocation to cellular membranes^[2]. However, stable targeting within cells is challenging. In particular, proteins localized at the plasma membrane undergo fast lateral diffusion^[3]. Here we report on a novel strategy termed “Molecular Activity Painting” to enable rapid, local and persistent activity perturbations at the plasma membrane by photoactivatable chemically induced dimerization (pCID) on immobilized artificial receptors. In contrast to photosensitive proteins, which require persistent illumination at wavelengths that also excite common fluorescent proteins^[4], this method is based on a single uncaging step at 405nm and thereby enables combined light-induced perturbation and imaging of a wide range of fluorescent proteins inside living cells.

Caged chemical dimerizers can induce stable protein dimerization after uncaging by a single illumination pulse^[5]. However, small molecules diffuse with a diffusion constant of several hundred $\mu\text{m}^2/\text{s}$ in the cytosol^[6] and thus distribute evenly in less than a second within a cell. Covalent linkage of a caged dimerizer to a membrane-anchored protein would reduce the diffusion constant by about three orders of magnitude^[3], thereby slowing down diffusion to a time scale of minutes. Therefore, we developed a cell permeable 6-nitroveratroyloxycarbonyl (Nvoc)-caged dimerizer (NvocTMP-Cl, **Scheme 1A**). The chlorohexyl moiety of the NvocTMP-Cl dimerizer binds covalently to a HaloTag domain that is targeted to the plasma membrane by an artificial receptor or a K-Ras-derived membrane anchor (CAAX). Photouncaging of the Nvoc group at 405nm enables the interaction between the trimethoprim (TMP) group and E. coli dihydrofolate reductase (eDHFR) fused to a protein of interest (POI). Recruitment of this fusion protein from the cytosol to the plasma membrane is measured via total internal reflection fluorescence (TIRF) microscopy and activation of effectors at the plasma membrane affects cell phenotype (**Scheme 1B**). By immobilizing the HaloTag via surface-bound artificial receptors we essentially abolish diffusion of dimerizers (**Scheme 1C**). NvocTMP-Cl is readily prepared in only four steps from readily available commercial materials, which facilitates its application in chemical biology studies (**Scheme S1**).

We first characterized NvocTMP-Cl mediated light-induced dimerization by targeting cytosolic eDHFR to HaloTag fused to the mitochondrial localization sequence of the *Listeria* ActA protein. Illumination with a 405nm laser rapidly induced protein targeting to mitochondria within 5 seconds, which was readily released within 2 min by adding the competitor TMP, highlighting the reversibility of the system (**Figure S1**). Importantly, NvocTMP-Cl showed negligible cytotoxicity up to 25 μM (**Figure S3**). Thus, we have established a rapid, bioorthogonal, reversible and easily implementable photo-induced dimerization system. During the course of our work, another group employed a related molecule for translocating proteins to intracellular structures^[7]. In our work, we aimed to control signaling processes at the plasma membrane, which acts as the central signal hub of cells.

We reasoned, that a dimerizer anchored to the PM might enable the generation of a transient dimerization gradient after local photouncaging. The resulting activity gradient could then control cell phenotype, such as directed cell migration. To test this idea, we used TIRF microscopy to analyze targeting of mCitrine-eDHFR to a plasma-membrane associated HaloTag fusion protein (TagBFP-HaloTag-CAAX). After treatment with 10 μM NvocTMP-Cl, we observed a very rapid recruitment of mCitrine-eDHFR to a region at the plasma membrane that was illuminated with a focused pulse of a 405nm laser ($t_{1/2} = 48$ ms, **Figure S4A-B and Supplementary Movie 1**). Previous CID and caged CID systems reported much slower targeting rates with $t_{1/2}$ between $\sim 15\text{s}$ and several minutes^[2c, 7]. To directly compare targeting via our system to the previously published caged CID system^[7], we measured $t_{1/2}$ of mitochondria targeting under comparable conditions i.e. identical fusion protein constructs and cell line, using scanning confocal microscopy. We obtained $t_{1/2} = 0.64 \pm 0.07\text{s}$, which is >20 times faster ($t_{1/2} \sim 15\text{s}$)

(**Figure S2**). We speculate that this improvement arises from the PEG-based linker, which may make the dimerization more favorable (**Scheme 1A**).

Next, we examined whether targeting of a signaling molecule to a plasma-membrane-anchored dimerizer can be used to locally control a biological process in cells. Rac1, a Rho GTPase family member, is known to stimulate the formation of thin, actin-based cell protrusions, called lamellipodia^[8]. To control Rac1 activity, we targeted a constitutively active Q61L mutant, which is lacking its C-terminal CAAX membrane anchor (mCitrine-eDHFR-Rac1Q61LΔCAAX, abbreviated as Rac1 perturbation) to the plasma membrane via local photouncaging ($t_{1/2} = 0.78$ s **Figure S4C-D** and **Supplementary Movie 1**). Although this mutant does not require upstream activators, full activation and induction of cell protrusions requires localization to the plasma membrane, where it can interact with its effectors to stimulate actin polymerization (**Scheme 1B**)^[9]. Although the local enrichment of active Rac1 attenuated within seconds due to diffusion, a transient gradient of protein targeting formed over several minutes by repeated laser pulses (**Figure S4E-G**, and **Supplementary Movie 2**). This gradient induced localized cell protrusion near the site of photouncaging (**Figure S4G-H**).

While multiple pulses of light were sufficient for transient targeting to a subregion of the plasma membrane, lateral diffusion limits the precision of local targeting. To suppress lateral diffusion, we anchored the HaloTag to artificial receptors, which were immobilized by their interaction with surface-bound antibodies (**Figure S5A-B**). Those receptors were derived from our previously developed bait-presenting artificial receptor constructs (bait-PARCs)^[10]. Here, we name these receptors dimerizer-presenting artificial receptor constructs (dimerizer-PARCs). We first optimized the processing of dimerizer-PARCs by modifying linker regions, introducing positively charged amino acids near the transmembrane domain and including a glycosylation site in the extracellular domain (**Figure S6**). Importantly, this dimerizer-PARC system is bioorthogonal: HaloTag derived from a bacterial alkyldehalogenase is not present in mammalian cells, and the TMP moiety binds to eDHFR with >1000 fold selectivity over mammalian DHFR^[9]. Furthermore, the artificial receptors are based on domains that do not interact with known signaling proteins and their cognate antibodies are directed against viral epitopes, which are not found in uninfected cells^[10].

Via the high affinity antibody-epitope interaction, the dimerizer-PARCs were strongly enriched on spots ($\phi=1.5\text{-}2\mu\text{m}$) of surface-bound antibodies (**Figure S5B**). Illumination of individual spots led to localized and sustained targeting of active Rac1 (**Figure S5B-D**, and **Supplementary Movie 3**) and co-targeting of the GTPase binding domain of PAK (**Figure S5G-H**). In contrast to targeting to a membrane anchored dimerization partner, no signal loss and no signs of diffusion were observed on immobilized receptors and no recruitment was observed a few micrometers away from the point of illumination (**Figure S5B-C, E**). This shows that the antibody-epitope interaction efficiently immobilized the dimerizer-PARCs and the associated dimerization reaction. One limitation of this method is that surface immobilization of dimerizer-PARCs also increases cell adhesion. We therefore suggest to use freely diffusing HaloTag-CAAX to study dynamic cell protrusion retraction processes as shown in **Figure S4**.

To test, if cell phenotype can be perturbed, we targeted the active C53R mutant of the Rho-specific activator GEF-H1 (*ARHGEF2*) to immobilized receptors. The local increase in Rho activity triggered by GEF-H1 translocation can be detected using a Rho activity sensor (**Figure 1A**). Furthermore, active Rho is expected to activate non-muscle myosin 2 via the Rho effector ROCK^[11]. Activated myosin 2 would associate with actin filaments in the local surrounding cell cortex and induce local contraction of acto-myosin structures by its motor function (**Figure 1A**).

To make stable targeting simpler and more flexible in those experiments, we used surfaces that were homogeneously functionalized with immobilized antibodies (**Scheme 1C**). This variant does not require specialized surface patterning devices and in addition allows “painting” of desired patterns of molecules in the plasma membrane via localized illumination. Here, persistent GEF-H1 targeting to illuminated spot regions lead to significant local Rho activation (**Figure 1B-D** and **Figure S7A-D**) and local

recruitment of non-muscle myosin 2a (*Myh2*) (**Figure 1E**, **Figure S7E-H** and **Figure S8A,C-D**). This recruitment was accompanied by contractile flow of the cell cortex towards the site of GEF-H1 targeting (**S7B-C** and **Supplementary Movie 4**).

Interestingly, while the levels of targeted GEF-H1 remained nearly constant after illumination, the Rho activity and myosin 2 response varied considerably from cell to cell, showing either a transient response or a sustained increase (**Figure 1C**, **Figure S7B,F,I,J**). This suggests that cell autonomous regulatory processes can modulate GEF-H1-RhoA-myosin 2 signaling. Importantly, the fast onset and long-lasting persistence of the switch-like perturbation enables a straightforward interpretation of the cellular response kinetics, which is invaluable for studying many plasma membrane associated processes, such as growth factor signaling, vesicle formation/fusion or turnover of other cytoskeletal structures. In particular, the fast onset is important to avoid cellular adaptation before maximal perturbation and the long-lasting persistence is important to reveal not only short-term but also long-term local adaptive responses. Here, without the inhibition of lateral diffusion via dimerizer-PARCs, the rapid attenuation of local perturbations ($t_{1/2} \sim 2\text{min}$; **Figure S5F**) would mask the highly variable Rho activity and myosin 2 adaptation responses (Rho activity: $t_{1/2}$ ranging from 20s to $>5\text{min}$, **Figure 1C** **Figure S7B**; Myosin recruitment: delayed response and subsequent adaptation ranging from 2min to $>20\text{min}$; **Figure S7F**, **Figure S8**, **Figure 1F** and **Supplementary Movie 4**). Furthermore, the combination of local persistent GEF-H1 perturbation and Rho activity measurements enables direct observation of reaction-diffusion based activity gradient formation (**Figure 1B**).

Next, we combined fixed local spot illumination with automated movements of the microscopy stage to “paint” arbitrary patterns of GEF-H1 activity, including letters and symbols with a line width down to $\sim 1\mu\text{m}$ (**Figure 1F**, **Figure S9** and **Supplementary Movies 5-6**). Those patterns were stable up to several hours (**Figure S9A-C**). Interestingly, we found that stable “painting” of GEF-H1 perturbation patterns directed the formation of new dynamic myosin structures (**Figure 1F-G**, **Figure S9E** and **Supplementary Movies 5-6**). For example, painting a $\sim 1\mu\text{m}$ wide line of active GEF-H1 perpendicular to preformed myosin-based stress fibers induced the formation of a dynamic, linear, myosin-based structure that coincided with the GEF-H1 activity pattern (**Figure S9E**).

In Molecular Activity Painting, we rapidly induce a switch-like perturbation by a single, focused pulse of 405nm laser light. This is an advantage compared to methods that use reversible photosensitive proteins, such as the PhyB-PIF system, which require constant patterned illumination to control their state^[4a, 12]. In the case of the PhyB-PIF system this is experimentally very challenging and requires simultaneous spatio-temporal control of two laser lines (650nm and 750nm) to actively switch the interaction on and off, for example via a digital micromirror device. The LOV1 and Cry2 domains are controlled by just one wavelength (typically 451nm or 488nm) and they spontaneously switch off in the dark. Thus spatio-temporal control is experimentally less complicated, but the spatial precision is limited by diffusion^[13]. Diffusion can be reduced via oligomerization of Cry2-based photosensitive proteins, however in this system, the spatial precision is limited by the uncontrolled formation of clusters and the onset of perturbations is considerably slower with halftimes in the range of several seconds^[14]. Molecular activity painting is performed with the commonly available 405nm laser line and patterning can be performed simply by simultaneously moving the automated microscopy stage as shown in this work. We also demonstrate that light-based protein targeting can be controlled by surface patterning techniques (**Figure S5**), which in principle enable structuring down to the nanometer scale^[15]. Therefore, our novel strategy also opens up new avenues to dynamically control the assembly of nanostructures inside living cells. Another advantage of Molecular Activity Painting is the activation wavelength: Photosensitive proteins display broad absorption spectra (PhyB: 550-800nm; LOV1: 400-500nm; Cry2: 390-500nm), which overlap with fluorescent proteins that are used to read out targeting dynamics and cellular responses. In our work, uncaging at 405nm was combined with measurements of four fluorophores, including mTurquoise2 (ex@451nm), mCitrine (ex@514nm), mCherry (ex@561nm) and

Atto740 dye (ex@750nm), which would be difficult using photosensitive proteins. A disadvantage of Molecular Activity Painting is the reversibility by competitor addition, which is slower than in PhyB-PIF, LOV1 or Cry2 domain-based systems..

In conclusion, we developed a rapid ($t_{1/2}$ in the range of milliseconds), bioorthogonal, reversible and readily implementable light-induced dimerization system. Using this system, we control the directionality of cell motility. By combining this system with dimerizer-presenting artificial receptors constructs, we establish a novel technology, “Molecular Activity Painting” (MAP) that enables switch-like, patterned perturbations of regulatory networks with micrometer precision to manipulate cellular signal processing and cell function at the plasma membrane of living cells.

Acknowledgements

Martin Lukas Kares and Darius Kaszta for assistance in artificial receptor optimization. Ola Sabet and Philippe Bastiaens for helpful discussions. Philippe Bastiaens for departmental support. Sven Müller for expert microscopy. This work was funded by the FORSYS partner initiative of the German Federal Ministry of Education and Research (BMBF, grant 0315258) to L.D.; the Deutsche Forschungsgemeinschaft, DFG (grant No.: SPP 1623 and SFB 642), Behrens Weise Stiftung and European Research Council, ERC (ChemBioAP) to Y.W.W., and a DAAD-Siemens fellowship, an IMPRS fellowship and a DAAD-PhD scholarship to M.V. CMN and MH acknowledge support by the Helmholtz programme BioInterfaces in Technology and Medicine and the Karlsruhe Nano Micro Facility (KNMF).

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Scheme 1. Design of the NvocTMP-Cl dimerizer system and its implementation in “Molecular Activity Painting” (MAP).

Figure 1: “Molecular Activity Painting” of arbitrary patterns at the plasma membrane. **A:** Contractility signaling and its manipulation via MAP. Plasma membrane targeting of the active GEF-H1 C53R mutant (GEF-H1 perturbation) triggers a signal cascade that leads to activation of the small GTPase RhoA and plasma membrane recruitment of myosin (Rho activity and myosin 2 response) and cell contraction. **B:** Representative example of local Rho activation via GEF-H1 recruitment to immobilized artificial receptors. **C:** Stable GEF-H1 perturbation at the plasma membrane resulted either

in a prolonged (top) or transient (bottom) Rho activity response. Black arrows in **B** indicate region for measurements shown in top panel of **C**. **D-E**: Quantification of maximal Rho activity and myosin 2 response in the presence or absence of photodimerizer (*:p<0.05; **:p<0.01; Student's t-Test; the number of analyzed cells is indicated below each column; data was obtained from 3 independent experiments). **F**: GEF-H1 painting of the letter "N" via ~1µm wide lines of active GEF-H1 induced localized accumulation of myosin in the proximity of the perturbation pattern. **G**: STICS flow analysis of myosin contractile movements (see also Supplementary Movie 6).