FINAL REPORT

1 General Information

DFG reference number: STI 656

Project number: 6-1

Project title: Sense and switch across scales – Prototyping genetically encoded, reversibly switchable indicators for sub-diffraction microscopy and whole animal optoacoustic Ca^{2+} imaging

Name(s) of the applicant(s): Andre C. Stiel

Official address(es): Helmholtz Munich, Ingolstädter Landstraße 1, 85764 Oberschleißheim

Name(s) of the co-applicants: n.a.

Name(s) of the cooperation partners: Dierk Niessing, Ulm/Munich, Vasilis Ntziachristos, Munich, (*planned*) Andriy Chmyrov, former Munich

Reporting period (entire funding period): entire funding period

2 Summary

Photoswitching proteins are key components in several high-resolution fluorescence microscopy techniques and also find application in signal unmixing in high-background scenarios (e.g. optoacoustic imaging or multiphoton microscopy). It is therefore surprising that the concept of photoswitching has not been associated with another important class of tools in modern life science imaging: genetically encoded sensors. Genetically encoded sensors for small molecules and ions enable the observation of the dynamic physiology of cells or tissues by visualising the temporal and spatial distribution of molecules and ions of interest. The combination of both concepts can have various applications ranging from fluorescence super-resolution microscopy of the distribution of small molecules or ions in the cell to interrogating cells deep in the tissue for their small molecules or ions homeostasis.

In the funded work we have succeeded in developing the first green fluorescent, photoswitching Ca^{2+} sensor, which shows that the concept is indeed realisable (published in *Nature Biotechnology*). With this sensor, we were able to show that super-resolution fluorescence microscopy can be performed to visualise Ca^{2+} distribution at the nanoscale. However, it also became clear that this first version of the sensor is yet not bright enough to allow reliable routine imaging – requiring further efforts. Additional to our protein engineering developments, we have elucidated the switching mechanism of the sensor at the molecular level by studying several structures of sensors and variants in the different switching states.

Moreover, we used the developed green sensor to demonstrate the general feasibility of using photoswitching sensors in optoacoustic imaging. We imaged mammalian cells expressing the sensor and implanted into an animal *in vivo* using optoacoustics. Unmixing was only possible for cells in the presence of Ca^{2+}. This demonstrates the feasibility of the switching sensor concept to detect molecule or ion concentrations in tissue, nonetheless, developments are necessary for variants specifically tailored to in tissue imaging.

Photoswitching-Proteine sind Schlüsselkomponenten in mehreren hochauflösenden Fluoreszenzmikroskopietechniken und finden auch Anwendung bei der Signalentmischung in Szenarien mit hohem Hintergrund (z. B. optoakustische Bildgebung oder Multiphotonenmikroskopie). Es ist daher überraschend, dass das Konzept des Photoswitching nicht mit einer anderen wichtigen Klasse von Tools in der modernen biowissenschaftlichen Bildgebung in Verbindung gebracht wurde: genetisch kodierte Sensoren. Genetisch kodierte Sensoren für kleine Moleküle und Ionen ermöglichen die Beobachtung der dynamischen Physiologie von Zellen oder Geweben durch die Visualisierung der zeitlichen und räumlichen Verteilung von Molekülen und Ionen. Die Kombination beider Konzepte kann verschiedene Anwendungen finden, die von der Fluoreszenz-Superauflösungsmikroskopie für die Visualisierung der Verteilung kleiner Moleküle oder Ionen in der Zelle bis zur Visualisierung der zellulären Homöostase tief im Gewebe reichen.

In der geförderten Arbeit ist es uns gelungen, den ersten grün fluoreszierenden, photoswitching Ca^{2+}-Sensor zu entwickeln, der zeigt, dass das Konzept tatsächlich realisierbar ist (veröffentlicht in Nature Biotechnology*). Mit diesem Sensor konnten wir zeigen, dass mit der superauflösenden Fluoreszenzmikroskopie die Ca^{2+}-Verteilung auf der Nanoskala sichtbar gemacht werden kann. Es wurde jedoch deutlich, dass diese erste Version des Sensors noch nicht hell genug ist, um eine zuverlässige Routinevisualisierung von Ca^{2+}-Verteilungen zu ermöglichen, und daher weitere Arbeiten erfordert. Neben der Entwicklung des ersten photoswitching Ca^{2+}-Sensors haben wir den Schaltmechanismus des Sensors auf molekularer Ebene aufgeklärt, indem wir mehrere Strukturen von Sensoren und Varianten in den verschiedenen Schaltzuständen untersucht haben.*

Unter Nutzung dieses grünen Sensors haben wir auch die generelle Machbarkeit des Einsatzes von Photoswitching-Sensoren in der optoakustischen Bildgebung nachgewiesen. Wir haben Säugetierzellen, die den Sensor exprimieren und in ein Tier implantiert wurden, in vivo *mittels Optoakustik abgebildet. Eine Entmischung war nur für Zellen in Gegenwart von Ca^{2+} möglich. Das Resultat zeigt das grundsätzliche Funktionieren des schaltenden Sensorkonzepts zur Erkennung von Molekül- oder Ionenkonzentrationen im Gewebe, nichtsdestotrotz sind noch weitere Arbeiten nötig, um Sensoren zu entwickeln die für die Nutzung im Gewebe optimiert sind.*

3 Progress Report

3.1 Short overview

The overarching idea of the funded research was **to explore the possibility of a photoswitchable small molecule / ion sensor** - switchable only in the presence of the molecule / ion of interest. Such a sensor could be employed in imaging schemes exploiting photoswitching. For example, **super-resolution fluorescence microscopy**, where photoswitching allows to restrict the spatial extent of fluorescence emissions to achieve sub-diffraction resolution[1], or recently emerging **photoswitching photo- / optoacoustics** where the photoswitching serves to create a modulation and separate the signal of the cells labeled with the switchable agent from the invariant background (mainly blood hemoglobin)[2].

Next to myriads of specialized solutions two larger concepts of building protein-based sensors for optical imaging exist[3]. First, FRET based sensors, where the binding of a molecule of interest changes the conformation of the binding protein. The changed confirmation results in a changed orientation of a suitably attached FRET pair increasing (or reducing) the Donor-Acceptor energy transfer. The second approach relies on **chimeric proteins where a binding protein (binding moiety) is directly attached to a readout protein (e.g. GFP, readout moiety)** and the conformational change upon binding is passed on directly to the chromophore environment altering its photophysics. For our proposed aim we pursued the second approach due to the possibility to **directly affect the photophysical properties of the chromophore, hence, likely also allowing to confer or prohibit photoswitching**.

Based on this concept two approaches are possible towards building first proof-of-concept sensors: i) building a sensor based on a photoswitching protein as readout moiety or ii) **using an existing sensor and introducing photoswitching capability by mutation in the readout moiety**. Our prototype existing at the beginning of the funding period was relying on the latter approach. We used the well know and studied GCaMP class of calcium sensors based on calmodulin and M13 (or RS20) as binding moiety and a circular permuted GFP as readout moiety[4]. The GFP used in GCaMPs shows high homology with photoswitching protein rsEGFP[5]. For the first prototype (rsGCaMP0.9) we inserted mutations differing between the GFP used in GCaMP5g and rsEGFP. The sensor rsGCaMP0.9 showed the general feasibility of the concept by exhibiting photoswitching with 488 and 405 nm light only in the presence of Ca^{2+}. However, rsGCaMP0.9 showed only low brightness (0.3) and switching dynamic range (70%). Hence, the goals of the funded work was to develop improved versions of rsGCaMP0.9 (T2.1), characterize those variants *in solution* (T2.3) and in mammalian cells (T3.1), perform super-resolution Ca^{2+} imaging with an improved version of the sensor (T4.1) and elucidate the structural details underlying the photoswitching sensor mechanism (WP5). **We were successful in establishing improved variants of rsGCaMP0.9 showing higher brightness (9.2) and switching dynamic range (90%) using those variants in collaboration with the Testa lab (Stockholm) we conducted super-resolution Ca^{2+} imaging in the ER. Furthermore, we elucidated the structures of several variants a pinpointed the mechanistic details including unusual chromophore conformations. The results have been published in *Nature Biotechnology*[6]** (see section 3.2.1 of this report, funding of this grant was used to advance the work in its late stages, while the first authors (K. Mishra and JP Fuenzalida Werner) were differently funded).

Despite those successes it became apparent that also the newer sensor variants exhibited a **photon budget below established super-resolution proteins like rsEGFP2**[7], hence requiring further development regarding the brightness of rsGCaMPs. Interestingly, parallel to our work another version of a photoswitching Ca^{2+} sensor was published[8]. This sensor was used for absolute Ca^{2+} concentration determination in cells. Despite of very similar building concept (GCaMP with photoswitching mutations) this sensor showed a much higher brightness (~30) than rsGCaMP1.4ER, our brightest version (~9.2). However, the sensor also showed a comparably low dynamic range of only ~80%, which leaves ~20% of residual fluorescence impeding an effective use in super resolution microscopy. We conducted a **mutational and screening study aiming to identify a version joining the benefits of both sensors' brightness *and* high switching dynamic range**. While not being able yet to establish a superior version of rsGCaMP, **we generated several interesting variants which displays interdependencies**

between brightness and switching dynamic range and chart the mutational environment of this novel sensor class (*unpublished*). Furthermore, since those photoswitching GCaMP sensors are the first sensors utilizing a circular permuted GFP in the context of photoswitching we elucidated in **structural and photophysical studies based on rsEGFP2 how the loss of the residues in beta strand 7** (missing in cpGFP) are impacting the photoswitching characteristics (*unpublished*).

Photo- / Optoacoustic imaging (OAI) is an *in vivo* in tissue imaging method which joins optical excitation with ultrasound detection. This allows **imaging far beyond fully optical methods (>> 1 mm) with comparably high resolution (<< 200 µm)**[9,10]. OAI already showed promising results in imaging e.g. the vessel infrastructure, in bio-medical imaging based on the strong contrast obtained from blood hemoglobin. However, OAI fell short for life-science imaging, because the targeted contrast from few labeled (e.g. transgene) cells of interest is easily concealed by the strong signal of the blood hemoglobin background. This challenge was approached by the introduction of photoswitching OAI (psOAI). In psOAI a photoswitching label (e.g. photoswitching protein) is used as targeted contrast agent and illumination with a given pattern of photoswitching wavelengths creates a **modulation of the signal from the labeled cells easily separatable from the invariant background by locked-in detection** and other concepts[2]. While this concept has been already demonstrated for the visualization of cell populations deep in the life animal (e.g. [11-14]) it so far did not exist for the use of molecular sensors to interrogate the small molecule and ion dynamics of cells in tissue. Using the photoswitching GCaMP rsGCaMP0.9 in our preliminary work, relying on *in solution* measurements we could already demonstrate that sensor unmixing in psOAI is conceptually feasible. The aim of the funded research was to demonstrate the concept *in vivo* (T3.3) and to develop a prototype sensor based on near-infrared (NIR) Bacteriophytochromes as readout moiety whose NIR absorbance are highly beneficial for deep tissue imaging (T2.2). **We were successful in demonstrating the functionality of the photoswitching sensor concept for psOAI *in vivo* using rsGCaMP1.1 an evolved version of rsGCaMP0.9 with higher switching dynamic range and absorptivity both beneficial for the unmixing in psOAI. Using rsGCaMP1.1 expressed in HeLa cells and implanted in a life mouse imaged using psOAI we could demonstrate by treating the implants with Ca^{2+} or with Ca^{2+} chelator EGTA that unmixing based on presence or absence of Ca^{2+} is possible in tissue *in vivo* (published in *Nature Biotechnology*[6], see section 3.2.4 of this report).**

While the demonstration with rsGCaMP1.1 was successful the green absorbance of this photoswitching sensor severely limits application in depth. Hence, we pursued **construction of sensor variants based on binding moiety Calmodulin/RS20 and NIR absorbing photoswitching BphP ReBphP-PCM**[11] as readout moiety. Initially we constructed several variants scouting potential insertion positions of RS20/Calmodulin into ReBphP-PCM. Some insertion positions resulted in non-folding or non chromophorylated variants while others showed expression with folding of ReBphP-PCM and attachment of the Biliverdin (BV) chromophore. Among the latter most of the variants showed normal photoswitching of the ReBphP-PCM, however no sensitivity to changing Ca^{2+} conditions. Suggesting an integration of the binding moiety RS20/Calmodulin in a position not “communicating” with the readout moiety. Another potential explanation is improper folding of the RS20/Calmodulin prohibiting Ca^{2+} binding. One variant in a similar position as was shown for non-switching NIR Ca^{2+} sensors[15] showed an **effect of the Ca^{2+} concentration on the photoswitching behavior of ReBphP-PCM in solution (rsRedGeci1, *unpublished*).** However, **characterization of the sensor in mammalian cells showed no signal suggesting an improper chromophorylation**. Moreover, studies based on the apo-form of the sensor showed a protein prone to aggregation suggesting a stabilization of the fold only by attachment of the BV chromophore. In mammalian cells this can additionally lead to a large fraction of unfolded protein if chromophore attachment and folding are not in conjunction leading to the net result of a sensor non-functional in mammalian cells. Along with WP5 we aimed at elucidating the structure of the sensor using crystallization for X-ray crystallography as well as CryoEM, however, so far without success.

Lastly, a study of the parent ReBphP-PCM also showed non-perfect chromophorylation in mammalian cells. In summary, this roadblock led to a shifting of our strategy for the generation of a BphP based sensors to focused on a **mutational study of the ReBphP-PCM pocket** aiming to identify mutations related to chromophore attachment and influence on the general photophysics. This study conducted

on **ReBphP-PCM produced a comprehensive mapping of the ReBphP chromophore environments along with several highly interesting photophysical variants, among them the brightest and most red-shifted BphP (and hence fluorescent protein) existing as well as a photoswitching version retaining fluorescence which is of high interest for super-resolution microscopy using BphPs** (BIORXIV/2026/708993). Based on the identification of a ReBphP variants without the PHY domain but retaining photoswitching we reapproached the sensor construction in the hope that the less complex environment in the chromophore surrounding due to the lack of the PHY-togue should facilitate chromophorylation, however, to no avail. Despite not managing to produce a photoswitching NIR Ca^{2+} sensor functional in mammalian cells, **the prototype rsRedGeci1 and the mutational study lay the groundwork for an efficient further exploration of NIR photoswitching sensors**. Lastly, the expertise of the student on the project on the ReBphP protein let to a contributing authorship in a study that aimed to use psOAI and ReBphP as injectable agent in translation efforts (*Acta Biomaterialia,* IF9.6)[16].

Finally, all efforts regarding the green sensor screening and characterization as well as the red sensor analysis have been flanked by the **development of screening and spectroscopic characterization equipment** (WP1).

1 Eggeling, C., Willig, K. I., Sahl, S. J. & Hell, S. W. Lens-based fluorescence nanoscopy. *Q Rev Biophys* **48**, 178-243 (2015). https://doi.org:10.1017/S0033583514000146

2 Stiel, A. C. & Ntziachristos, V. Controlling the sound of light: photoswitching optoacoustic imaging. *Nat Methods* **21**, 1996-2007 (2024). https://doi.org:10.1038/s41592-024-02396-2

3 Mo, G. C. *et al.* Genetically encoded biosensors for visualizing live-cell biochemical activity at super-resolution. *Nat Methods* **14**, 427-434 (2017). https://doi.org:10.1038/nmeth.4221

4 Akerboom, J. *et al.* Optimization of a GCaMP calcium indicator for neural activity imaging. *J Neurosci* **32**, 13819-13840 (2012). https://doi.org:10.1523/JNEUROSCI.2601-12.2012

5 Grotjohann, T. *et al.* Diffraction-unlimited all-optical imaging and writing with a photochromic GFP. *Nature* **478**, 204-208 (2011). https://doi.org:10.1038/nature10497

6 Mishra, K. *et al.* Genetically encoded photo-switchable molecular sensors for optoacoustic and super-resolution imaging. *Nat Biotechnol* **40**, 598-605 (2022). https://doi.org:10.1038/s41587-021-01100-5

7 Grotjohann, T. *et al.* rsEGFP2 enables fast RESOLFT nanoscopy of living cells. *Elife* **1**, e00248 (2012). https://doi.org:10.7554/eLife.00248

8 Bierbuesse, F. *et al.* Absolute measurement of cellular activities using photochromic single-fluorophore biosensors and intermittent quantification. *Nat Commun* **13**, 1850 (2022). https://doi.org:10.1038/s41467-022-29508-w

9 Ntziachristos, V. Going deeper than microscopy: the optical imaging frontier in biology. *Nat Methods* **7**, 603-614 (2010). https://doi.org:10.1038/nmeth.1483

10 Wang, L. V. & Hu, S. Photoacoustic tomography: in vivo imaging from organelles to organs. *Science* **335**, 1458-1462 (2012). https://doi.org:10.1126/science.1216210

11 Mishra, K. *et al.* Multiplexed whole-animal imaging with reversibly switchable optoacoustic proteins. *Sci Adv* **6**, eaaz6293 (2020). https://doi.org:10.1126/sciadv.aaz6293

12 Yao, J. *et al*. Multiscale photoacoustic tomography using reversibly switchable bacterial phytochrome as a near-infrared photochromic probe. *Nat Methods* **13**, 67-73 (2016). https://doi.org:10.1038/nmeth.3656

13 Li, L. *et al*. Small near-infrared photochromic protein for photoacoustic multi-contrast imaging and detection of protein interactions in vivo. *Nat Commun* **9**, 2734 (2018). https://doi.org:10.1038/s41467-018-05231-3

14 Märk, J. *et al*. Dual-wavelength 3D photoacoustic imaging of mammalian cells using a photoswitchable phytochrome reporter protein. *Communications Physics* **1**, 3-3 (2018). https://doi.org:10.1038/s42005-017-0003-2

15 Qian, Y. *et al*. A genetically encoded near-infrared fluorescent calcium ion indicator. *Nat Methods* **16**, 171-174 (2019). https://doi.org:10.1038/s41592-018-0294-6

16 Huang, Y. *et al*. Photoswitching protein-XTEN fusions as injectable optoacoustic probes. *Acta Biomater* **195**, 536-546 (2025). https://doi.org:10.1016/j.actbio.2025.02.002

4 Published Project Results

4.1 Category A – Articles in peer-reviewed journals, contributions to peer-reviewed conferences or to anthology volumes, and book publications

- Mishra K, Fuenzalida-Werner JP, Pennacchietti F, Janowski R, Chmyrov A, Huang Y, Zakian C, Klemm U, Testa I, Niessing D, Ntziachristos V, Stiel AC.. Genetically encoded photo-switchable molecular sensors for optoacoustic and super-resolution imaging. ***Nat Biotechnol***. 2022 10.1038/s41587-021-01100-5

Works partially funded by the project. Funding acknowledged.

- Huang Y*, Stankevych M*, Gujrati V, Klemm U, Mohammed A, Wiesner D, Saccomano M, Tost M, Feuchtinger A, Mishra K, Bruns O, Geerlof A, Ntziachristos V, Stiel AC.. Photoswitching protein-XTEN fusions as injectable optoacoustic probes. ***Acta Biomater***. 2025 10.1016/j.actbio.2025.02.002, * shared first authorships

Underlined author (Mohammed) funded by the project. Funding acknowledged.

- Rodrigues EC, Stiel AC.. It's a two-way street: Photoswitching and reversible changes of the protein matrix in photoswitchable fluorescent proteins and bacteriophytochromes. ***FEBS Lett***. 2023*10.1002/1873-3468.14609*

Contents of the review driven by research from the funded project. Funding acknowledged.

- Stiel AC, Ntziachristos V.. Controlling the sound of light: photoswitching optoacoustic imaging. ***Nat Methods***. 2024 10.1038/s41592-024-02396-2

Contents of the review driven by research from the funded project. Funding acknowledged.