Projet de Recherche Doctoral Concours IPV 2021

Intitulé du Projet de Recherche Doctoral :

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Unité de Recherche :
Intitulé : PASTEUR
Code : UMR 8640
Equipe de Recherche *(au sein de l'unité)* **:**
Intitulé : Pôle de chimie physique et biologique de la matière vivante
Thématique de recherche : Imagerie sélective exploitant la réactivité des marqueurs
Responsable d'équipe :
NOM : JULLIEN Prénom : Ludovic
Ecole Doctorale de rattachement de l'équipe & d'inscription du doctorant : ED 388 (Ecole doctorale de Chimie Physique et de Chimie Analytique de Paris Centre)

Doctorants actuellement encadrés par le directeur de thèse (préciser le nombre de doctorants, leur année de 1ere inscription et la quotité d'encadrement) : Une étudiante en thèse, Aliénor Lahlou, co-encadrée à 30% (avec Thomas LE SAUX, Maître de conférences, Sorbonne Université, pour 70%) et inscrite à l'ED 388 en février 2020 (contrat CIFRE).

<u>CO-DIRECTION</u> *(obligatoire)*
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Unité de Recherche :
Intitulé : Biologie cellulaire et cancer
Code: UMR 144
Equipe de Recherche *(au sein de l'unité)* **:**
Intitulé : Dynamique de l'organisation intracellulaire
Thématique de recherche : Développements technologiques appliqués à l'étude de la dynamique de l'appareil de Golgi et des mécanismes régulant la dynamique des microtubules
Responsable d'équipe :
NOM : PEREZ Prénom : Franck
Ecole Doctorale de rattachement : ED 515 Complexité du vivant

Doctorants actuellement encadrés par le co-directeur de thèse (préciser le nombre de doctorants, leur année de 1ere inscription et la quotité d'encadrement) : Un étudiant en thèse, Octave JOLIOT, inscrit à l'ED515 "Complexité du Vivant" en Novembre 2020 (contrat ANR).

Cotutelle internationale : ☒ Non ☐ Oui, précisez Pays et Université :

Précisez ici les éventuels co-encadrants (non HDR)

Co-encadrant :

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Unité de Recherche :

Intitulé : PASTEUR

Code : UMR 8640

Equipe de Recherche *(au sein de l'unité)* **:**

Intitulé : Pôle de chimie physique et biologique de la matière vivante

Thématique de recherche : Imagerie sélective exploitant la réactivité des marqueurs

Responsable d'équipe :

NOM : JULLIEN — Prénom : Ludovic

Ecole Doctorale de rattachement : ED 388 (Ecole doctorale de Chimie Physique et de Chimie Analytique de Paris Centre)

Résumé (2 000 caractères maximum) :

Tags become essential for biomolecular labeling. In fluorescence microscopy, fluorescent proteins (FPs) enabled the selective labeling of proteins through genetic fusion. Later on, (ir)reversibly photoswitchable proteins have considerably expanded the range of applications of FPs. First used in subdiffraction optical imaging techniques, they have been then used for contrast enhancement by combining optically driven fluorescence modulation with signal demodulation methods. In particular, we introduced several protocols for selective imaging of reversibly photoswitchable fluorescent proteins (RSFPs) under adverse optical conditions.

Although FPs provide powerful labels for imaging proteins, they experience limitations, which have motivated the development of chemogenetic reporters. In particular, we developed the FAST tag, which forms non-covalent fluorescent assemblies with non-fluorescent synthetic dyes. In this project, we will overcome the FAST approach by developing protein tags combining fluorogenicity and photochromism (rpFAST). Giving access to unprecedented non-covalent RSFPs absorbing/emitting light over a wide range of wavelengths and photoswitching over a wide range of time scales, they will enable for highly multiplexed fluorescence imaging, even in strongly autofluorescent biological samples (e.g. transparized tissues).

We will exploit a similar strategy to introduce a second series of non-covalent reversibly photoswitchable protein tags (SAST: Sound Activated and absorption-Shifting Tag) for photoacoustic microscopy, which can image tissues well beyond the penetration limit of fluorescence microscopy. In this microscopy modality, they will open a way for highly multiplexed imaging by combining two mechanisms allowing for time-gated detection of the photoacoustic signal: fast selective thermoelastic expansion at the protein tag from red-shifted absorbance of its dye, and slower electrostriction after photoisomerization-induced dye ejection from the tag.

Joindre en annexe un descriptif du PRD avec références au format pdf (« NOM_2_IPV_2021 » / 3 pages maximum, taille police 11)

AVIS et VALIDATION de l'ECOLE DOCTORALE :

Le projet de thèse proposé est en adéquation avec les thématiques de l'école doctorale 388.
Mr Ludovic Jullien dirige actuellement une thèse rattachée à l'ED 388 dont la soutenance est prévue en 2022.
L'ecole doctorale émet ainsi un avis favorable pour cette demande de financement de thèse au prés de l'IPV.

à envoyer simultanément par e-mail à l'ED de rattachement et au programme : interfaces_pour_le_vivant@listes.upmc.fr avant le lundi 15 février minuit.

MULTICOLOR NON-COVALENT REVERSIBLY PHOTOSWITCHABLE PROTEIN TAGS FOR MULTIPLEXED OBSERVATIONS UNDER ADVERSE OPTICAL CONDITIONS IN FLUORESCENCE AND PHOTOACOUSTIC IMAGING

Context and objectives

Our understanding of cells and organisms relies on observing how their constituents organize and interact. Optical microscopy techniques have become essential in biology as they allow to study the dynamics of labeled structures and biomolecules with high spatial and temporal resolution. In fluorescence microscopy, the green fluorescent protein (GFP) and its analogs (FPs) have enabled the selective labeling of proteins through genetic fusion.[1] Later on, the development of irreversibly or reversibly photoswitchable proteins able to switch from dark to bright states or from one color to another upon light irradiation has considerably expanded the range of applications of conventional FPs.[2] First implemented to develop subdiffraction optical imaging techniques (such as PALM[3] or STORM[4]),[5] the photoactivable fluorophores have been then used for contrast enhancement by combining optically driven fluorescence modulation with various signal demodulation methods.[6-9]

In particular, we introduced the (Speed)OPIOM imaging protocols, which reveal the kinetics of the photochemical reactions of fluorescent labels by a modulated illumination.[9-11] These protocols enabled us to selectively discriminate the fluorescence signal of reversibly photoswitchable fluorescent proteins (RSFPs) under adverse optical conditions. Notably, we imaged RSFPs against autofluorescence (from the biological sample or from a polymeric membrane for Western blots), ambient light (up to sunlight), or spectrally similar non-photoswitchable fluorophores. Moreover, Speed-OPIOM significantly pushed forward the multiplexing capability by discriminating four spectrally similar green RSFPs upon matching the frequency and intensity of modulated illumination with the photoswitching kinetics. More recently, we introduced two novel protocols, which further boosted multiplexed fluorescence imaging:[12,13] twenty spectrally similar green RSFPs have been discriminated at 1 Hz acquisition frequency after simple and robust processing of their fluorescence signal collected upon applying appropriate sequences of illumination.

Although FPs provide powerful labels for imaging proteins in live cells, they experience limitations that prevent their unrestricted use in molecular imaging.[14] Their size and tendency to oligomerize can lead to dysfunctional fusions, their long fluorescence maturation time can prevent the study of dynamic processes, their limited photostability prevents long-term tracking, and their need for molecular oxygen prevents imaging under anaerobic processes. In the case of RSFPs, the available library is further restricted to three families sharing the same spectral channels and photochemistries,[15] which we showed to limit the number of distinguishable RSFPs by our imaging protocols.

Such challenges have motivated the development of new reporters made of a genetic tag that selectively binds synthetic fluorophores.[16] These chemogenetic reporters combine the targeting selectivity of genetic tags with the advantages of organic synthesis, which can yield fluorophores exhibiting higher brightness and photostability, and be tuned at will by molecular engineering. Hence, we developed a small monomeric protein tag (FAST: Fluorescence-Activating and absorption-Shifting Tag).[17] It forms non-covalent fluorescent assemblies with substituted 4-hydroxybenzylidene rhodanine derivatives, which essentially exhibit no detectable fluorescence in solution or in cells. Hence, selective imaging of FAST-tagged proteins can be achieved without the need for washing the free ligand in excess, facilitating the study of dynamic processes and the imaging of multicellular organisms. More recently, FAST has been evolved to yield a same protein tag allowing the genetic encoding of blue, cyan, green, yellow, orange and red fluorescence via non-covalent binding of different fluorogenic dyes.[18] In this project, we will considerably enlarge the scope of the FAST fluorescence turn-on approach by developing protein tags combining fluorogenicity and photochromism (rpFAST). They will yield non-covalent RSFPs absorbing/emitting light over a wide range of wavelengths and endowed with exclusive contrast between their reversibly photoswitched states so as to be highly attractive for multiplexed fluorescence imaging even under adverse optical conditions as well as for subdiffraction microscopy (Figure 1A). In particular, we will especially focus on fluorescence imaging of transparized biological samples. First, our design of non-covalent protein tags is compatible with fast post-transparization tag labelling with small fluorogenic dyes, which will overcome transparization-induced bleaching limitations commonly encountered with regular fluorescent proteins. Second, contrast enhancement relying on reversibly photoswitchable tags will eliminate the interference from autofluorescence of the transparized samples.

Dual systems made of a protein scaffold non-covalently binding a chromophore could also be exploited in photoacoustic microscopy[19-21] for *in vivo* imaging well beyond the penetration limit of fluorescence microscopy, which is restricted to superficial observation of tissues by light scattering even with confocal and multiphoton microscopies.[22] Photoacoustic microscopy relies on resolving the origin of ultrasonic waves generated by absorption of photons at a chromophore.[23] Ultrasonic waves are scattered much less than light in tissue, which enables to break the optical diffusion limit. Photoacoustic microscopy usually exploits the thermoelastic expansion following transient local temperature rise owing to molecules that absorb energy from light. However, this mechanism can only provide a modest level of multiplexed observation by relying on spectral discrimination. As in fluorescence microscopy, RSFPs have been used to overcome this limitation by exploiting the time evolution of their photoacoustic signal upon photoswitching.[24,25] Reversibly photoswitchable non-fluorescent proteins have been subsequently introduced to optimize the signal-to-noise-ratio.[26] In this project, we want to go one step further by introducing non-covalent reversibly photoswitchable non-fluorescent protein tags absorbing light at several wavelengths and combining two mechanisms enriching time-gated detection of the photoacoustic signal (SAST: Sound Activated and absorption-Shifting Tag; Figure 1B): (i) fast thermoelastic expansion selectively occurring at the protein tag from red-shifted absorbance of its non-fluorescent chromophore and (ii) slower electrostriction after photoisomerization-induced chromophore ejection from the tag, which will cause a volume

change and a pressure wave as a consequence of the significant alteration of the organization of the water solvation shell around the light absorber and the protein tag.[27]

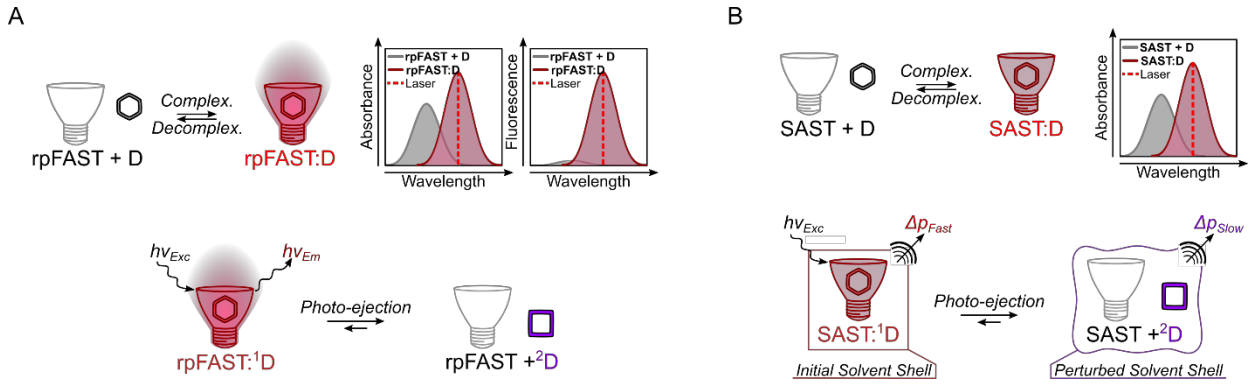

Figure 1. rpFAST (A) and SAST (B) for multiplexed observations under adverse optical conditions in fluorescence and photoacoustic imaging. The complexation of the dye within the cavity of the protein tag generates a red shift for selective recovery of the protein tag (with simultaneous enhancement of the dye brightness in A). Upon illumination, the complexed dye experiences reversible photoisomerization, which disrupts the dye-protein tag complex. The specific time evolution of the collected signal is used for kinetic discrimination and selective imaging.

Scientific program

A collection of dyes. The first part of this project will deal with the design, the synthesis, and the photophysical/photochemical investigation of a collection of dyes. A common list of specifications is relevant to address both fields of application relying either on fluorescence imaging or photoacoustic imaging:

- In order to permeate towards the targeted cells, the dyes should be not too large and water-soluble to cross cell membranes, but not too hydrophobic neither to limit trapping within cell membranes;

- The dyes must bind their protein scaffolds. In order to maintain significant exchange dynamics to avoid any detrimental photobleaching, we will target dye-protein complexes typically exhibiting 0.1-1 μM dissociation constants;

- The dyes must reveal their protein scaffolds and only them. To work with this range of affinity necessitates to work at least in the presence of 0.1-1 μM dye concentrations to get significant occupation of the labeling protein scaffold, with the risk to reveal non-specific complexes of intermediate stability. Hence, we will request the desired complexation event to specifically red-shift the light absorption of the dye, which will enable for selective recovery of the protein tag signal by tuning the excitation wavelength to the red edge. Two different strategies are considered for red-shifting the absorption spectrum of the dye in its bound state: (i) Negative solvatochromism. The binding of the dye within the hydrophobic scaffold cavity is anticipated to be associated with a drop of polarity of the dye environment. Therefore, we will engineer dyes exhibiting a larger dipole moment in their first excited state than in their ground state in order to drive a red-shift in absorption by the transfer from water to a more hydrophobic cavity; (ii) Change of ionization of the dye upon binding. We will engineer dyes, which experience a change of their ionization state (e.g. mediated by proton exchange) upon binding in an appropriate protein scaffold. For applications in fluorescence imaging, we will further get a second discriminative tool by requesting complexation to enhance the dye brightness from motion restriction;

- Upon illumination, the chromophore will experience reversible photoisomerization, which will disrupt the chromophore-protein tag complex. It will result in a specific time evolution of the collected signal, which will be used for kinetic discrimination.

Following this list of specifications, we have first selected a series of donor-acceptor conjugated dyes bearing a phenol as an ionizable electron-donating group conjugated to an electron-withdrawing group by means of an aromatic ring and a double bond. Based on our previous experience in the FAST series,[18,28] we already identified several molecular backbones, which exhibit cis-trans photoisomerization and absorb in the 400-550 nm range. To further expand the range of absorption wavelength of the dyes towards infra-red which is favorable for deep imaging in tissues, we also selected a series of Donor-Acceptor Stenhouse Adducts,[29] which are subjected to a large reorganization upon photoisomerization exchanging a highly conjugate apolar state absorbing beyond 600 nm which reminds retinoids (to be selectively complexed within the protein scaffold) and a colorless state smaller in size and more polar absorbing below 400 nm only (anticipated to be the stable state in water). After their syntheses, these dyes will be assayed *in vitro* (both in aqueous and organic solutions, as well as in cultured cells) to evaluate whether their physicochemical properties fulfil the list of requested specifications.

A collection of protein scaffolds. The second part of this project deals with the design of protein scaffolds, which we constrained to fulfill two criteria: (i) To be monomeric and small in order to be active as fusion protein and not perturb the function of the protein they are fused to; (ii) To fold and be active no matter of the redox cellular conditions to enable use in any cell compartments.

In order to address the size issue and to obtain a tag working in any cell compartments, we propose to evolve small monomeric protein binders able to fold properly in reducing conditions. We will focus our engineering effort on nanobodies. These binders are generally robust and intrabodies, i.e. antibodies folding in cell cytosols, have been developed. In particular, we identified a robust nanobody scaffold that was then used to generated diverse libraries.[30]

To generate protein scaffolds able to induce the desired alteration of a dye, we will rely on directed protein evolution by applying an iterative Darwinian optimization process with steps of mutation, selection and amplification to select the fittest variants from an

ensemble of random mutations.[31] To do so, we will use phage-displayed libraries of 10^9 nanobodies with randomized complementarity determining regions. Biopanning using magnetic beads functionalized with the dyes of interest will enable to select binders and eliminate non-functional nanobodies Among the selected binders, those forming red-shifted (for both fluorescence and photoacoustic imaging applications) and fluorescent (for fluorescence imaging applications) assemblies with the dyes will be further characterized. Additional rounds of maturation may eventually be performed using libraries of yeast displayed nanobodies, which allow screening for fluorescence properties using high-throughput fluorescence-activated cell sorting (FACS).[17]

Validation of the non-covalent reversibly photoswitchable protein tags. The best tag candidates will be evaluated to image and quantify proteins with our imaging protocols for contrast enhancement in fluorescence and photoacoustic (collaboration with Prof. Emmanuel Bossy, University of Grenoble) imaging. Beyond validation in cultured cells, organoids, and model organisms (e.g. zebrafish) with both imaging modalities, we will then focus on fluorescence imaging of transparized biological samples (slices of mice brain and chicken embryos; collaboration with Susanne Bolte, in charge of the cellular imaging facility at IBPS, FR 3631, Sorbonne Université). In particular, we will screen several transparization protocols to evaluate the applicative scope of the developed non-covalent RSFPs for fluorescence imaging of transparized tissues and organs.

Presentation of the consortium

The project consortium involves complementary partners, who already collaborated and published together.

The **UMR 8640 partner** (Ludovic Jullien, PR SU; Thomas Le Saux, MC SU - who received in 2018 the Instrumentation Prize from the French Societies of Physics and Chemistry and who will get his HDR in Spring 2021) from the group of physical and biological chemistry of living matter, laboratory PASTEUR (UMR CNRS-ENS-SU 8640, Paris) has expertise in organic syntheses of photoactive probes, photophysics/photochemistry, analytical chemistry, fluorescence imaging, and chemical thermo-kinetics (including thermal aspects relevant for photoacoustic imaging).[9-13,17,18,28,32]

The **UMR 144 partner** (Franck Perez, DR CNRS) is the Chairman of the Cell Biology and Cancer Unit at Institut Curie. His group is studying intracellular protein transport with a strong investment in technological development. In particular, his group created a quantitative transport assay usable for real-time imaging and screening. It also developed the use of recombinant antibodies in cell biology studies[33,34] and recently focused on nanobody development.[30,35,36]

This consortium may also benefit from the collaboration with Arnaud Gautier (PR SU, Laboratoire des Biomolécules, UMR CNRS-SU-ENS 7203) if directed evolution would have to rely on yeast display.

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[1] Overlined references originate from the consortium members.