FRANCE-BIOIMAGING

Roadmap

for next generation imaging technologies

2023 - 2030

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Roadmap for next generation imaging technologies (2023-2030)

To develop the next generation imaging methods, we identified five strategic priorities for instrumentation, and two accompanying priorities, for probe development and image processing (including deep learning). Each priority is supervised by a dedicated Working Group (WG), which provides a national supervision for technological developments and implementations, as well as a forum for R&D exchanges, technology watch activities, and tools to transfer R&D innovations to facilities.

Our five strategic priorities are:

1. 1. *Super-resolution and single molecule tracking*
2. 2. *Multiscale correlative microscopy (including EM and AFM)*
3. 3. *Multiscale light sheet microscopy*
4. 4. *New contrast and in-depth imaging, preclinical microscopy*
5. 5. *High-content and multiplexed imaging*

These are supported by two accompanying priorities:

1. 1. *BioImage informatics (data management, visualization and analysis, including deep learning)*
2. 2. *Probe development*

Strategic Priority n°1 - Super-resolution and single molecule tracking

Super-resolution microscopies (SRM) were awarded the Nobel prize in chemistry in 2014. These technologies have revolutionized cell biology, developmental biology and neurosciences. SRM makes it possible to monitor the nanoscale organization and dynamics in living organisms, with down to single molecule resolution. FBI has been very active in this area, and some of our recent methodological developments include: (i) super-resolution in high-content screening formats (Beghin, et al. 2017, Nature Methods); (ii) correlative single molecule & STED microscopy (Inavalli, et al. 2019, Nat Methods); (ii) nanometric axial resolution with modulated excitation (Jouchet, et al. 2021, Nature Photonics); (iii) single molecule microscopy on large fields of view (Mau, et al. 2021, Nature Communications); labeling and analysis methods, including a very popular tessellation-based method for colocalizing single molecules (Bessa-Neto, et al. 2021, Nature Communications).

However, despite spectacular improvements over the last decade, super-resolution still remains limited in speed of data acquisition, in its ability to investigate 3D biological samples, and in the quantitative analysis that can be achieved. To further improve, SRM would strongly benefit from interdisciplinarity in the fields of chemistry, optics and computer sciences. In particular, to enable novel breakthroughs in single molecule biology, there is a need to attain to following aims:

Aim 1: Develop new probes based on small tags and high labeling efficiency, attached with fluorophores with improved photophysics compatible with SRM, but also new self-blinking dyes compatible with live cells to probe functional imaging at the nanoscale. These probes will be developed in collaboration with the R&D chemistry teams of France-BioImaging (see *Accompanying Priority n°2* below).

Aim 2: Develop and implement new instrumentation: (i) to improve the resolution in all spatial dimensions; (ii) to allow in-depth multi-proteins nanoscopy that benefits from alternative optical sectioning, wavefront optimization for aberration-controlled imaging, stochasticity and targeted light-modulation; (iii) to reveal new parameters at the nanoscale such as orientation through polarized fluorescence (Vaz Rimoli, et al. 2022, Nature Communications), spectrum, lifetime, pH…

Aim 3: Implement computational approaches, and recent deep-learning in particular, to tackle the issues that cannot be overcome physically or by traditional approaches, with the goal to first optimize the image acquisition beyond optical limits (higher resolution, lower noise, faster acquisition and more colors), and second provide the community with dedicated quantitative analysis tools. Computerized methods will be jointly developed with the IPDM working group (see below *Accompanying Priority n°1*).

Strategic Priority n°2 - Multiscale correlative microscopy

Electron microscopy (EM) and cryoEM methods (2017 Nobel prize in Chemistry) have witnessed a spectacular development in the last decade, both in terms of resolution (covering a large range of structural analysis from mm to sub-nanometer-scale in 2D and 3D), and in the high-throughput automation of data acquisition. In addition, correlative approaches (CLEM) have been developing to place molecular information obtained from light microscopy in the structural cellular context obtained by EM. Overall, EM and light microscopy combined in CLEM methods have become incredibly powerful tools to investigate living organisms. Here, FBI's developments for instance succeeded in creating new models enabling SRM, EM and dynamic light microscopy in intact tissue (Franke, et al. 2019, Traffic; Chambaud, et al. 2022, Plant Physiol,;Getz, et al. 2022, Science Advances; Figon, et al. 2021, Proc Natl Acad Sci U S A).

With the development of super-resolution light microscopy techniques with close to nanometer resolution (STED, STORM), the relevance of correlation between EM and light microscopy is increasing, therefore allowing correlations at a higher resolution, at the level of molecular complexes.. A good example lies in combining the power of the specificity of fluorescence microscopy together with the ultrastructural contextualization information provided by EM. We will continue to work actively in CLEM developments. **One of the main challenges is to combine these levels of resolution** in order to visualize exactly the same event in time and space and at different levels of resolution. Not surprisingly, the main objectives and milestones are thus on par with SRM developments. We propose the following aims in this axis:

Aim 1: Development and testing of new probes, labels and sample preparations amenable to CLEM approaches. The 3 main challenges are: a) the development and use of probes suitable both for SRM and EM as described above (*Strategic Priority n°1*); b) the development of sample preparations adapted to CLEM, for example the generalization of Cryo-FIB-SEM to obtain samples adapted for cryo-tomography; c) the capacity of using efficient light microscopy probes in correlative workflows with electron microscopy. The exploitation of SRM at low temperatures correlated with Cryo-EM will for example be developed within the Equipex+ NanoCryoClem (Bordeaux).

Aim 2: Scouting and implementation of developments in CLEM. CLEM approaches are developing rapidly worldwide, with developments both in **live-CLEM** (temporal correlation), **spatial CLEM (2D or 3D)** and **cryo-CLEM**. We will track and promote innovative developments and resources for CLEM approaches (instruments, expertise, software) through extensive networking, exchanges with industrials and survey of the equipment and software available at various FBI facilities in France. The new approaches will be implemented in particular on the 3D-EM microscopes which are available in Marseille, Bordeaux and the new cryoTEM which was recently purchased with FBI funding and implemented in Paris-Centre (PICT Curie, mostly for cryoET; Ultrapole Pasteur, mostly for cryoCLEM; Imagoseine Institut Jacques Monod, mostly for room-temperature 3D-CLEM).

Aim 3: Image processing for CLEM and 3DEM. FBI members of the BI-IPDM node have already developed renowned approaches to easily correlate multimodal images (eC-CLEM; Paul-Gilloteaux, et al. 2017, Nat Methods) and deep-learning methods in Cryo-EM for automatic identification of macromolecules in cryo-ET volumes, down to 550 kDa (DeepFinder; Moebel, et al. 2021, Nat Methods). The next developments will be in linking multidimensional SRM with CryoEM.

Strategic Priority n°3 - Multiscale light sheet microscopy (LSM)

Biological mechanisms and processes occur at scales ranging from molecules to tissues and at speeds from milliseconds to days, and accessing the relevant information requires a variety of microscopes able to cover diverse regions of the volume/resolution/speed multidimensional space. **LSM has not only low phototoxicity, but also allows accessing novel regions of this space, thereby providing entirely new sets of information on living systems**. Because of this unique potential, LSM technologies have witnessed spectacular developments in the last few years. FBI has been very active in this area, and recent advances for instance include multiphoton LSM technologies, with the the demonstration of

two-photon imaging at ~500 Hz frame rate (Maioli, et al. 2020, Biomedical Optics Express), and fast live imaging of SHG nanoparticles (Malkinson, et al. 2020, ACS Photonics). Other spectacular developments include the creation of an automated high-speed 3D imaging system for organoids phenotypic screening at multiple scales (Beghin, et al. 2022, Nat Methods). Developing LSM microscopies will thus be an essential part of our action and LSM developments will pursue three goals:

Aim 1: LSM of large specimen, by developing multiphoton non-linear LSM (Ile-de-France-Sud, Marseille).

Aim 2: LSM at high-speed, high-resolution, by combining the FBI soSPIM technology with MFM (Paris Centre), Atomic Force Microscopy (Montpellier), or combining Adaptive Optics with LSM methods, soSPIM/LLSM-AO (Bordeaux). This also includes LSM technologies for high-content screening (Bordeaux).

Aim 3: Develop suitable image analysis and visualization tools (see below *Accompanying Priority n°1*), as LSM technologies produce extremely vast amounts of data not easily visualized and explored by users. Implementations in instrumentation will work hand-in-hand with the development of suitable analysis and visualization tools by the IPDM Node.

Strategic Priority n°4 - New contrast and in-depth imaging, preclinical microscopy

High-resolution 3D imaging of intact tissues is an important field of microscopy, progressing through the ongoing maturation of technological bricks (multiphoton lasers, adaptive optics, scanning geometries, etc.) and the validation of multiple and complementary contrast modalities (2PEF, 3PEF, SHG, THG, Raman, CARS/SRS, polarization, FLIM, etc). Besides these ongoing developments and the maturation of their known application fields (neuroscience, stem cell biology, etc), a timely and important topic is currently to apply these technologies to the context of translational and medical research. The development of microscopy for preclinical research represents a strong added value of FBI. By integrating in 2020 new facilities focused on this topic, **FBI now constitutes a full continuum from microscopy for fundamental research to preclinical approaches, opening new avenues of applications and interests in the areas of regenerative medicine, cardiovascular diseases, oncology and immunotherapy** to cite only a few. Here, FBI's current aim is to provide the latest technological and methodological advances in microscopy for an integrated understanding of cellular and tissue activities, in particular for preclinical research. Indeed, FBI combines cutting edge approaches of cellular imaging and histology on a plurality of experimental models (up to primates). Recent technological and applicative advances include the development of large-volume color serial two-photon microscopy ("ChroMS") (Abdeladim, et al. 2019, Nature Communications) and its application to cortical astrocytes lineage analysis (Clavreul, et al. 2019, Nature Communications); the introduction of a new contrast modality based on TSFG for in-depth label-free blood microscopy (Ferrer Ortas, Light Sci App, accepted); the application of long-term two-photon microscopy to study neural stem cell fate decisions in the adult fish brain (Dray, et al. 2021, Cell Stem Cell). Our future objectives and milestones will be to:

Aim 1: Develop and implement label free microscopy modalities enabling new optical contrast (Raman, IR, non-linear, OCT, Polarization, Quantitative Phase/Digital Holographic Microscopy/Ptychography combined with tomography) in the context of cellular and tissue imaging for preclinical studies;

Aim 2: develop multimodal (label free, bright field staining and fluorescence) **and automatisation** (slide scanner, automatisation…) **microscopy for histopathology** (fixed samples including very thick);

Aim 3: fill the gap between preclinical microscopy and medical imaging, by developing (i) intravital microscopy, (ii) organoid screening and (iii) the correlation between living sample and 3D reconstructed fixed thick samples (obtained from Aim 2). This strategy is totally in line with the activity of FBI, where technological developments are motivated by identified biological needs. By ensuring a continuum between biological imaging and medical imaging, we aim to develop a new line of services and methodological and technological transfer to users of microscopy for preclinical studies.

Strategic Priority n°5 - High-content and multiplexed imaging

High Content microscopy and its modern variants represent a unique approach to explore cellular processes and phenotypes. Two aspects are rapidly evolving in that area: (i) smart microscopy, where complex live cell imaging scheme combining multiple modalities can be implemented in an automated manner to scale-up the throughput, via on the fly analysis of pre-scan images; (ii) multiplexing techniques, which have seen an explosion in the last few years as they now enable to visualize up to 10,000 different molecules in single cells (e.g. so-called spatial transcriptomics, elected method of the year by Nature Methods in 2020; Marx 2021, Nature Methods). These technologies achieved a remarkable breakthrough, with great potential to open new venues to understand biological processes. These currently mainly focus in the gene expression field, but are expected to rapidly diffuse to many other areas of life sciences. FBI has been very active in this field, with the creation of intelligent imaging solutions (in part via the creation of the Inscoper start-up), and the development of high-throughput and/or multiplexed smFISH detection of RNAs and chromatin loci (Cardozo Gizzi, et al. 2019, Mol Cell; Espinola, et al. 2021, Nat Genet.; Chouaib, et al. 2020, Dev Cell.; Safieddine, et al. 2021, Nat Commun.). Dedicated image analysis workflows have also been developed by teams of the IPDM Node (Samacoits, et al. 2018, Nature Communications; Mueller, et al. 2013, Nature Methods). Our future objectives will build on these advances to:

Aim 1: Develop and implement smart microscopy techniques in FBI facilities, for using complex acquisition schemes in medium to high-throughput.

Aim 2: Develop new methods to improve the throughput of current multiplexed technologies and extend them to the acquisition of thick specimens.

Aim 3: Combine multiplexed detection of RNA, chromatin and proteins with other imaging methods (super-resolution, live cell imaging, etc.), or orthogonal approaches (i.e. sequencing-based spatial transcriptomics, single cell proteomics). Indeed, single cell biology is a flourishing field, and by combining diverse imaging modalities with biochemical approaches, FBI will bring a truly unique contribution to this key field. We will leverage **machine learning / deep-learning approaches** to enable integrated structure/dynamics/function studies and to predict one modality from another.

Accompanying priority n°1 - BioImage informatics (IPDM Node)

Bioimage informatics has seen an explosive development in the last ten years, in large part because of the spectacular progress in computer vision and artificial intelligence, including deep learning. It is now an integral part of microscopy, sometimes directly incorporated in the instruments or co-conceived with the instrumental part. In the next 8 years, FBI will develop bioimage informatics in four areas: (i) artificial intelligence and image analysis; (ii) visualization of high-dimensional datasets; (iii) development of a remote image analysis service; (iv) management of image data.

To achieve these goals, the IPDM Node can rely on 7 core R&D teams and 10 additional teams located in the geographic Nodes. Moreover, FBI is implementing a team of data engineers (FBI.data team), involved in developing a remote image analysis service and helping core facilities to implement FAIR bioimage data management practices. Importantly, our institutional bodies (CNRS, INSERM, Universities) committed to provide stable positions for these engineers. The FBI.data team should have a total of 10 engineers (three have already been hired). The IPDM Node thus provides a very rich ecosystem for data management, analysis and visualization.

Aim 1: Artificial Intelligence and Image Analysis. Machine learning and computer vision are propelling a revolution in image data analysis. Many segmentation and classification tasks considered unfeasible a few years ago, can be solved today efficiently with neural networks. Artificial intelligence has also provided stunning results in image restoration and cross-modality predictions. As described in Section 2 (Impact), the IPDM Node has been very active in developing tools addressing many of these key issues in image analysis. In the future, two different aspects will be developed. **First**, IPDM R&D teams will continue developing tools addressing the needs of our Strategic Priorities, for instance, image restoration algorithms for single molecule microscopy, registration tools for correlative microscopy (including super-resolution CLEM), automated object/event identification by deep learning in LSM and

cryo-EM images (Serpico / Deep Finder · GitLab (inria.fr)), and cross-modality predictions in multiplexed microscopy. Most of these developments are already ongoing. **Second**, provide easy access to these tools to biologists. Indeed, many biologists have no access to these cutting-edge methods, because of the lack of interoperable, user-friendly software that can handle AI libraries and could be easily used by non-experts. **To provide access to these technologies**, we will: (i) integrate AI libraries in our generalist software platform Icy (de Chaumont, et al. 2012, Nat Methods), to make it AI-compatible; (ii) continue to develop open, AI-compatible software and gateways to build integrative analyses solutions (e.g. ImJoy (Ouyang, et al. 2019, Nat Methods.), Bioimage-IT (Prigent, et al. 2022, Nat Methods); (iii) provide human resources to transfer existing AI-based analysis tools into AI-compatible open software, middleware or platforms (e.g. ImJoy, Bioimage-IT, Icy, respectively), which are frequently installed at the facilities.

Aim 2: Visualization of high-dimensional image datasets. Nowadays, the detection and visualization of localized events and processes in 3D+time images are tedious and inefficient, especially in cell and tissue imaging. Scientists can miss key events due to the complexity of the data and the lack of computer guidance. Here, we plan to **develop original and cutting-edge visualization and navigation methods to assist scientists**. The next generation visualization software will allow a more efficient analysis and interactive exploration with a temporal series of multi-valued volumetric images, to better face the needs of modern imaging methods and in particular light sheet microscopy. We will expand recent advances in scientific visualization and Virtual and Augmented Reality (VR/AR), in order to provide visualization software and/or setups capable of assisting scientists for a better understanding of large 3D+Time movies or highly multiplexed images (where thousands of different molecules can be present). Specifically, we will aim at: **(i) implementing hardware tools for VR/AR**, such as digital holography (Hololens), visualization headsets, or the use of immersion chambers; **(ii) developing and implementing software tools for visualization**, including mathematical methods to encode and represent spatio-temporal information in VR, and computational methods to render point clouds and meshes; display several volumes (atlas, colocalization analysis, simulated versus real datasets) in VR; (iii) connect multiple users for interaction on the same volume in a VR experiment; (iv) interact with image databases to annotate training datasets for Deep Learning; (v) interact with generalist databases (Genebank, etc...), to display multiple information in VR. To this end, IPDM has formed a subgroup containing 5 R&D FBI teams as well as additional developers in France. This group has already organized web services, training sessions and recently published the perspectives brought by the new VR/AR approaches in the field of biological imaging (Valades-Cruz, et al. 2022, Front Bioinform).

Aim 3: Developing a remote image analysis service. The needs of biologists in image analysis are extremely acute and the implementation of the new technologies proposed here will only worsen the situation. To address this challenge and maximize the help provided to users, FBI has initiated an image analysis service accessible remotely (FBI-AS, led by JY. Tinevez, Pasteur, and AS. Macé, Curie). This service currently regroups 11 bioimage analysts present in the different Nodes of the infrastructure, which dedicate a part of their time to this service. FBI-AS is accessible to anyone via virtual open desk sessions, and it provides various services (free ~1h consultation of user projects -already implemented-; billable 5-25h analysis projects -planned for 2023). It is therefore a highly innovative initiative that has the triple advantage of: (i) giving access to advanced image analysis methods to any user; (ii) maximizing the output of bioimage analysts; (iii) creating an expert community and a rich scientific environment beneficial to all bioimage analysts. To further develop this service, we propose that the new bioimage analysts that will be recruited on FBI facilities over the years will be affiliated to the FBI-AS and will spend 20-40% of their time for this service. This will reinforce the service, allow the training of additional bioimage analysts on new technologies, and embed every bioimage analyst hired in a strong community that will be a driving force for everyone.

Aim 4: Management of image data. This is a pillar of our infrastructure action plan and it is conducted in close collaboration with the IFB National Infrastructure (Bioinformatics, see also section 7 below). We are engaged in the following: (i) develop and implement a Data

Management Plan for the infrastructure complying with FAIR recommendations, and which can be reused by other infrastructures using bioimaging (such as EMBRC, CELPHEDIA, *etc.*); (ii) install image management databases such as OMERO or equivalent, in all core facilities, both locally and in distant mutualized mesocentres; (iii) develop and implement distant image analysis and visualization softwares, and stimulate the use of distant mutualized resources; (iv) purchase storage and computing resources (local by us, and in distant mesocenters by IFB), such that image data produced by the infrastructure are traced through their life cycle, ensuring FAIRness of the data and implementation of machine-actionable Data Management Plans by IFB; (v) develop the use of public image repository for published data, by creating user-friendly links to public repository.

These actions are led by our two 'Data' Mission Officers, which work in close collaboration with the IFB. Moreover, we take into account the necessary consistency with international initiatives, in particular at the European level through our activities within ERIC-EuBI and ELIXIR.

Accompanying priority n°2: Probe development

The development of technologies to introduce non-invasive perturbations in living systems and probe the associated response remains a key issue of the field of bioimaging. New effectors beyond light (temperature, magnetic fields,...) have recently emerged for introducing perturbations. They involve new objects (e.g. nanomaterials) and new challenges (e.g. control over the stoichiometry of their derivatization). The development of novel libraries of exogenous probes is requested by the emergence of modalities of new contrasts (Raman, IR,TPEF, SHG, TGH, photoacoustic,...) as well as by the demand for information-rich probes reporting on a multitude of events instead of being only passive labels. FBI R&D teams have been very active to develop and characterize: (i) new probes and labels, enabling to overcome spectral limitations (Chouket, et al. 2022, Nat. Commun; Quérard, et al. 2017, Nat. Commun) or label and modify endogenous proteins in live cells (Benaissa, et al. 2021, Nat Commun), (ii) novel chemogenic fluorescent reporters with unique dynamic properties enabling for instance tunable color for advanced imaging or sensitive protein-protein interaction biosensing in living cells (Tebo, et al. 2019, Nat Commun); (iii) macroscale fluorescent imaging against autofluorescence and under ambient light (Gautier, et al. 2014, Nat Chem Biol). Our future objectives will be to:

Aim 1: Work in close interaction with Strategic Priority n°1 and n°2 (super-resolution and single molecule tracking; Multiscale and correlative microscopy), in order to develop and implement probes suitable for improved resolution, new super-resolution modalities with environmental sensitivity, EM/cryo-EM and super-resolution compatibility

Aim 2: Be an interface between the probe developers and the end-users by relying on the imaging facilities. In particular we will identify and support reference FBI facilities for evaluating new technologies**.**

Aim 3: Sustain a continuous scientific watch activity and organize hands-on trainings to favor dissemination of the most promising technologies.

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