Analyse de la dynamique par imagerie de FRAP-4D, ou 4D-FRAP imaging

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Résumé – Les Progrès réalisés dans le domaine de la microscopie et de la biologie cellulaire permettent aujourd'hui l'acquisition et l'analyse de l'activité cellulaire à haute résolution spatiale, spectrale et temporelle. Les techniques de photo-perturbation, telles que le FRAP, la photo-activation la photo-conversion ou la photo-ablation par exemple, permettent d'obtenir des informations complémentaires à l'échelle moléculaire. Malheureusement, elles restent séparées des systèmes d'imagerie rapide car elles ne sont accessibles que par l'intermédiaire de microscopes confocaux à balayage laser. Pour palier à cette limitation, nous avons développé un module permettant de combiner sur un même microscope un système de guidage laser avec une imagerie rapide champ large, à onde évanescente ou confocale multipoints.

1. Introduction

Thanks to the discovery and cloning of naturally fluorescent proteins, it became conceivable to directly follow cellular structures and their molecular constituents under a microscope. It is now clear that the spatio-temporal localization of molecular constructions within the cell and their dynamic responses to diverse physical or biomolecular perturbations are keys for the understanding of basic mechanisms in life sciences. At the moment, multidimensional (nD) data of living cells required for this type of analysis are generated by multiple sophisticated instruments. One of the major drawbacks of these microscopic tools resides in the lack of flexibility needed to monitor, influence, perturb and measure life material and its behaviour. Moreover, most image processing and analysis techniques that are accessible to the research community through available packages are insufficiently adapted. Finally, as the amount of collected data is becoming extremely rich of information, the interest in developing computer-based techniques to allow automatic analyses of this data corpus is obvious. For all these reasons, we and others are currently developing "multi-mode" microscopy combining high throughput nD microscopy with or without photonic perturbations and subsequent automated image analysis.

2. Fast nD-FRAP/PA Imaging microscopy

As an example of our strategy for challenging acquisition and analysis of complex cellular activities, we present a novel instrument that brings together the advantages of both laser-assisted techniques, for FRAP (Fluorescence Recovery After Photobleaching) or PA (Photo-Activation), and those of rapid multidimensional imaging. More precisely, it combines on the same microscope a position-controlled laser system with a fast 4-5D deconvolution microscope (Fig. 1A and 1C). The laser module is coupled to the rear port of a wide-field microscope through a specific dichroic mirror placed in the dual illumination port (Fig. 1B and 1C). This ensures maximum efficiency for both monochromator and laser (405nm, 488nm) illuminations. Optical paths for wide-field and FRAP/PA illuminations are separated and independent, allowing simultaneous laser injection and image acquisition.
FRAP/PA is performed by precisely focusing down a laser beam in the sample during a short duration. Regions of the sample to bleach/activate are selected in 3D by the user (Fig. 1D). In order to get an accurate matching between image coordinates and scanner positions on the whole field of the camera, an automatic calibration with a 4^{th} order polynomial fit is preliminary performed. This allows taking into account optical aberrations of the illumination path, especially distortion. We will first validate its performance, using quantitative analyses of diffusion measurements for two-dimensional lipid layers. We then apply this technique to living cells expressing Rab6-GFP, a protein cycling between a cytosolic pool and a membrane form partly concentrated on the inner face of the Golgi apparatus.

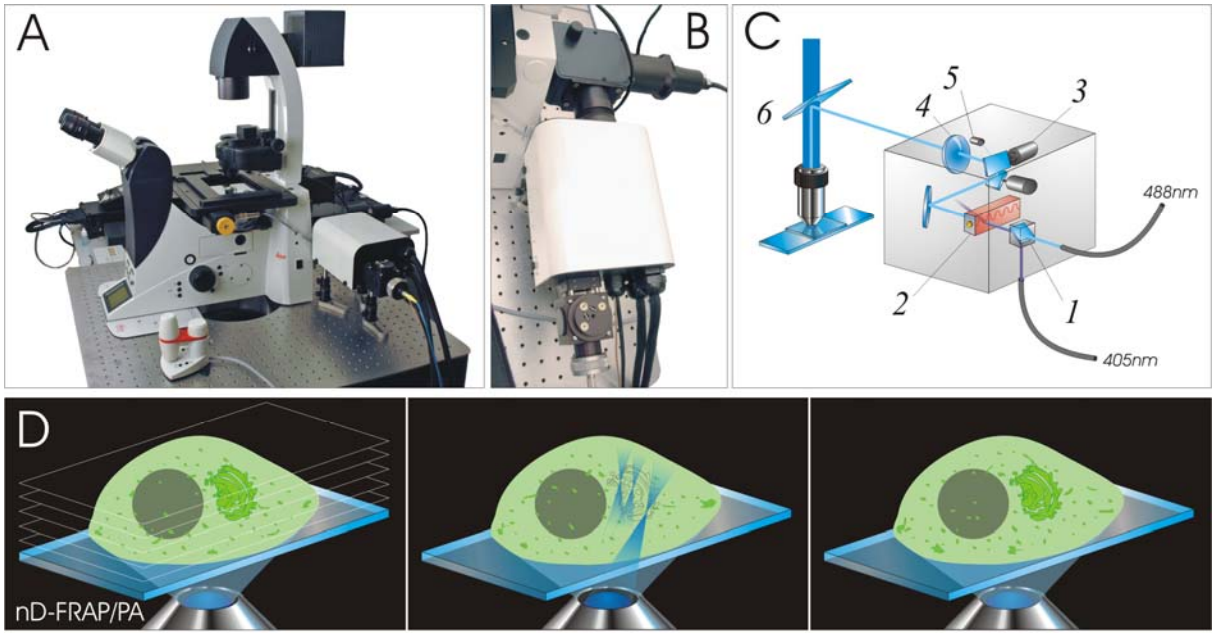

Figure 1 *– A) Overview of the Fast 4D-FRAP imaging system and B) the laser module. C) Schema of the laser module, with 1) Beam combiner, 2) AOTF, 3) Scanning mirrors, 4) Coupling lens, 5) Power measurement and 6) Coupling mirror. D) (3D+t)-FRAP/PA Imaging sequence is divided in 4 steps: 0- Setup of the acquisition parameters of pre-bleach, bleaching and recovery sequences and determination of the volume of interest to bleach/PA. 1- Pre-Bleach acquisition in 3D+t. 2- 3D positioning of the laser in the region(s) of interest determined in step 0 for Photobleaching or PA. 3- Post bleach/PA acquisition in 3D+t.*

3. Image Analysis

Beside measurements on 2D diffusion or biochemical exchange between cytosol and membrane associated molecules, full understanding of proteins dynamics in live cells often requires 3D imaging for the visualization of particular events, like membrane exchange. Dedicated tracking methods to study the movement of objects can then be performed. This is where nD-FRAP/PA Imaging associated with image analysis comes into play. Experimentally, the use of this methodology on 4D FRAP/PA Imaging data generated massive information, enlightening an unexpected heterogeneity of behaviours and allows statistical analysis of the contribution of vesicle movements to the cellular cycle of proteins.

4. Conclusion

We have developed optical tools (FRAP, PA) to perturb the cell activity equilibrium in combination with fast multidimensional microscopy and automated image analysis tools. Quantitative data collected in a very reproducible and normalized way can now be incorporated into statistical models. Ultimately we expect such a strategy to provide new models and concepts in life dynamics.