MICROSCOPY

Microscopy **62(1)**: 63–68 (2013)
doi: 10.1093/jmicro/dfs130

Review

Fluorescence imaging in the last two decades

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Abstract In commemoration of the 20th anniversary of the molecular cloning of the gene for the green fluorescent protein from the jellyfish *Aequorea victoria*, I would like to reflect on the development of new fluorescence imaging technology in the last two decades. As this technology has become increasingly diversified, it has become more and more of a challenge to come up with a comprehensive and exhaustive review of it. Here I will focus on optogenetics and large-scale, three-dimensional reconstruction. Those two technological innovations have been achieved in the neuroscience community owing to the combined efforts of molecular biologists and light microscopists. In addition, modern fluorescence imaging has indeed improved our understanding of the spatiotemporal regulation of fundamental biological functions at cellular level. As an example, I will introduce some findings we made regarding the movement of biomolecules across the nuclear membrane. The above-mentioned imaging approaches are possible today but were impossible two decades ago.

Keywords fluorescence, development, imaging

Received 15 November 2012, accepted 17 December 2012; online 7 February 2013

Introduction

The conventional techniques for labeling proteins with organic dyes for live imaging consist of laborious steps. Reliable protein labeling requires expertise in protein chemistry and the microinjection of labeled products into cells using glass pipettes with minimal cell damage requires much experience. The means of introducing fluorescence-labeled proteins directly into specific sites within a cell is limited because the distribution and targeting of most proteins are regulated by their translation and post-translational modifications. In contrast, *de novo* synthesis is much more likely to result in native patterns of localization. Gene transfer techniques, including the construction of transgenic organisms, liposome-mediated transfection, the use of various viral vectors, electroporation and the gene gun, have shown significant progress in recent years. Proteins can be expressed within cells as fusions to small tags that can react with specialized organic fluorophores [1–4] or to fluorescent proteins (FPs) that can become spontaneously fluorescent through the autocatalytic synthesis of a chromophore (below).

Green FP (GFP) was originally isolated from the light-emitting organ of the jellyfish *Aequorea victoria* by Shimomura *et al.* [5]. Approximately 30 years had passed before the complementary DNA encoding the protein was cloned by Prasher *et al.* [6] and subsequently characterized by Chalfie *et al.* [7]. Since then, our ability to unravel the fine details of cellular events has improved remarkably. Chimeric GFPs can be expressed *in situ* by gene transfer into cells and localized to particular sites with appropriate targeting signals [8]. Furthermore, the emergence of the spectral variants of GFP [8],

as well as GFP-like proteins from other organisms [9–11], has paved the way for investigators to observe simultaneously multiple cellular events. The family of GFP-like proteins, in particular, is greatly expanding and continuing to grow to this day. Although some GFP-like proteins exhibit striking features, they share a few basic properties [12]. Here, the term 'FPs' is used to describe genetically encoded fluorophores, including GFP-like proteins as well as *Aequorea* GFP and its variants.

Optogenetics

Outstanding advances in genome science and gene technology have led to numerous discoveries and the development of new technologies in life sciences. The new technologies include 'optogenetics' – a growing suite of techniques that combine optical and molecular genetic methods [13–15]. The new technologies are becoming popular particularly in neuroscience, where the central challenge is understanding the mechanisms by which neurons process and integrate synaptic inputs and how those mechanisms are modified by activity.

Optogenetic imaging with molecular sensors has great potential for investigations in neuroscience by virtue of its high spatial and temporal resolution. Researchers have studied both the biological and practical aspects of a variety of FPs with the goal of enhancing their biological properties and making them practically useful. Many genetically encoded sensors have been developed for key intracellular signaling molecules (events) [16], such as calcium ion (excitation) [17], membrane potential (excitation) [18,19], chloride ion (excitation) [20], pH (synaptic transmission) [21,22] and glutamate (neurotransmitter release/uptake) [23,24]. The sensors are used to investigate the function of specific signaling mechanisms in synaptic transmission, integration and plasticity, and to study neuronal firing inside the brain.

Optogenetic control of neuronal activity allows us to selectively activate or inactivate genetically defined populations of neurons in order to examine how the activity of neurons contributes to the function of neural circuits in the brain. For example, the light-activated ion channel channelrhodopsin-2 (ChR2) [25], originally found in the green algae *Chlamydomonas*, can be expressed in neurons, allowing brief flashes of blue light to activate the neurons. Likewise, the expression of the light-activated chloride pump, halorhodopsin (NpHR) [26], allows the inactivation of neuronal activity.

Such genetically encoded tools are introduced by gene transfer techniques into an intact organism and their expression is targeted to specific tissues, cell types or subcellular compartments, thereby allowing the efficient detection or manipulation of neuronal activity. Due to the recent groundbreaking progress in gene transfer techniques, including electroporation, viral-mediated gene transfer and germline transmission of transgenes, studies are not only limited to mice but can also be performed in primates [27].

The emergence of new optogenetic tools will surely stimulate the imagination of many neuroscientists. The demand for these tools and the aspirations of researchers who use them are sure to soar. As a result, fluorescence microscopes will inevitably have to be equipped with special hardware and software to make the best use of them. In this regard, a significant evolution of light microscopes will be necessary if optogenetic technologies are to enjoy widespread use. Commercial light microscopy (LM) systems should evolve into ones that are amenable to the addition of new functions.

Large-scale, three-dimensional (3D) reconstruction

One important advantage of FPs over organic dyes is their ability to be genetically introduced into biological tissues regardless of the depth of the target area. With the advent of transgenic techniques to label specific cells with FPs, life scientists are awaiting a new optical technique that can provide large-scale and finely detailed perspectives of labeled structures within a large biological specimen. In the neuroscience community, for example, it is difficult to produce high-resolution reconstructions of entire neuronal networks in fixed brain samples. Within the field, there is an escalating demand for new techniques that seek to address this issue, such as the development of Brainbow mice [28]. Such techniques are critical to comprehensive 'connectomic analyses' [29,30].

The three-dimensional (3D) imaging of large biological specimens involves sectioning in order to improve axial resolution. Mechanical sectioning methods allow efficient observations of genetically or immunohistochemically labeled structures with subcellular resolution, but are extremely laborious for large-scale 3D reconstruction unless used in conjunction with well-designed automation procedures, such as array tomography [31]. 3D reconstruction using electron microscopy (EM) ensures nano-scale resolution, but EM is generally applicable only to very small specimens. As recently developed techniques, such as the serial block-face scanning EM [32,33], have improved the quality of voluminous EM data and automated the data acquisition, it is expected that the technique can efficiently process large tissue volumes [34].

Some research groups have employed optical sectioning methods and explored a multitude of ways to achieve subcellular resolution in the 3D reconstruction of fluorescently labeled structures within large tissue samples, such as whole mouse brains. The cerebral cortex of an adult mouse brain is ~1 mm thick. Accordingly, if we intend to comprehensively survey labeled neuronal circuits that extend beyond the cortex, we need to examine brain specimens of millimeter-order thickness.

Successful optical imaging deep into tissue is hampered mostly by the scattering of light. Generally, a biological sample can be viewed only by laser scanning confocal microscopy (LSCM) to a depth of ~150 µm below the surface. To overcome the problem stemming from light scattering, two approaches may be useful. One approach is 2-photon excitation fluorescence microscopy (TPEFM). This approach greatly improves penetration into tissue because the fluorescence excitation is conducted with light in the near-infrared region, which is scattered less than visible light. Furthermore, because TPEFM does not use pinholes, the emitted photons can be collected with maximum efficiency even if they are scattered within a thick tissue [35,36]. However, very few scientists have achieved TPEFM imaging beyond 800 µm deep in intact brain tissue at high resolution [36]. Nevertheless, it should be noted that TPEFM could be used effectively to image live rather than fixed biological samples.

The other approach to overcoming light scattering is optical clearing, which aims to increase tissue transparency in order to achieve refractive uniformity throughout a fixed sample. The water-soluble reagent *FocusClear*™ is used to clear whole brains of insects [37], including cockroaches and fruit flies. Unfortunately, this commercial reagent is prohibitively expensive for large samples. Furthermore, because its contents are undisclosed, the properties of *FocusClear*™ are unknown, which makes it difficult to optimize clearing procedures for different biological samples. As a result, the use of *FocusClear*™ is limited mostly to entomological studies. In fact, there are no reports showing that the reagent can sufficiently clear mammalian brain tissue.

Optical clearing techniques have co-evolved with hardware and software advances. For example, one research group has developed an ultramicroscopy system to perform 3D reconstructions of large structures in whole organs of mice as well as fruit flies [38,39]. In this microscopy system, which adopts light sheet illumination, large fixed specimens are chemically cleared prior to image acquisition. To achieve chemical clearing, a fixed sample is incubated in an organic clearing solution of one part benzyl alcohol and two parts benzyl benzoate after dehydration with ethanol and hexane. Although this chemical clearing procedure substantially quenches FPs inside the specimens, a recent study has revealed that clearing with dibenzyl ether after dehydration with tetrahydrofuran is more FP-friendly [40].

My group has developed an aqueous clearing reagent that shows superb ability to clear tissue yet preserve the signals of FPs [41]. Remarkable improvement was achieved in the deep imaging of intact (un-sectioned) mouse brains in which yellow fluorescent protein was transgenically expressed in a subpopulation of neurons [42]. The neurons were clearly visualized to a depth of several millimeters below the brain surface (Fig. 1), not only with 2-photon (TPEFM) but also with single-photon (LSCM) approaches. Our study will dramatically advance the imaging depth limit, which is now primarily set by the working distances of objective lenses. In addition, sweeping views were obtained

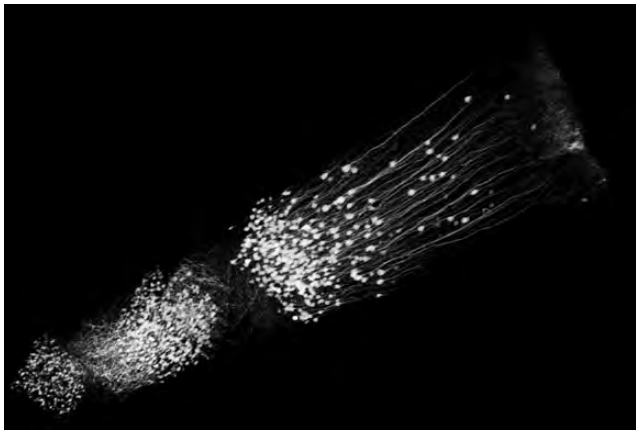

Fig. 1 3D reconstruction of yellow fluorescent protein (YFP)-expressing neurons in a quadratic prism located in the cerebral cortex and the hippocampus. Neurons in the intact brain of *thy1-YFP* mouse line H were visualized after fixation and a 2-week treatment with Sca*l*eA2.

of callosal axons that travelled long distances across the midline. Given its simplicity and stability, this reagent will popularize the technique of high-resolution 3D quantification of structures within intact organs and animals.

Translocation across nuclear envelope (NE)

Molecules for life are always on the move. The recent realization that the regulation of signal transduction is unexpectedly dynamic in space and time has spawned fervent interest in the diffusion, transport, fluctuation, and translocation of biomolecules. In the last decade, numerous new technologies that use FPs have been developed, allowing researchers to observe and manipulate the spatiotemporal dynamics of biomolecules in live cells. Following the emergence of photoactivatable FP [43] and Kaede [44] in 2002, a variety of photomodulatable FPs have been developed [12,45]. In each of those FPs, photoactivation or photoconversion is usually achieved by excitation of the protonated form of the chromophore that absorbs light maximally at ~400 nm. It is almost a coincidence that the Blu-ray Disc player market has grown in the last decade. As 405 nm Blu-ray diode lasers have become the standard light source in the industrial realm, today, most commercial CLSM systems are equipped with violet laser diodes that facilitate photoactivation/photoconversion techniques. There appear to be further tips and tricks in the techniques that use photomodulatable FPs. The accumulation of more feedbacks from users will refine the basic procedures and diversify the techniques even more.

Considerable progress has been made in detailing the fundamental mechanism underlying the compartmentalization of the nucleus. The nuclear envelope (NE) defines the nuclear compartment and nuclear pore complexes (NPCs) in the NE form aqueous passages through which small water-soluble molecules can passively diffuse. A mature nucleus is characterized by the exclusion of large diffusing molecules and the ability to import nuclear localization signal-containing molecules. However, there remain a number of unresolved issues regarding protein translocation between the nucleus and the cytoplasm. I will briefly introduce two of our studies that have addressed such issues.

Maturation of diffusion barrier

The development of a diffusion barrier composed of NE and NPCs around newly assembled nuclei was investigated. Researchers determined the effective sizes of those pores by introducing labeled molecules of different sizes into the cytoplasm and measuring their rates of diffusion into the nucleus. The experiments revealed that proteins <50 kDa diffused through the pores, whereas proteins >60 kDa rarely entered by passive diffusion. However, little is known about how this size cut-off value changes as the NE reassembles and the nucleus expands. My group [46] and Ellenberg's group [47] used KikGR [48] and Dronpa [49], respectively, to identify a short period very early in the G_1 phase (after cytokinesis), during which the NE barrier is more permeable than previously thought.

Speed of nucleocytoplasmic shuttling

The irreversibility of photobleaching/photoactivation techniques prevents recurrent monitoring of the same protein to study its temporal regulation. A monomeric FP that displays photochromism was developed and named Dronpa [49]. Dronpa absorbs light at 503 nm and emits bright green fluorescence. It can also be converted by strong irradiation at 488 nm into a non-fluorescent state absorbing at 390 nm, and then switched back to the original

emissive state with minimal irradiation at 405 nm. These photochromic characteristics enable studies of fast protein dynamics at multiple time points in individual cells. Through repeated labeling of the same regions, the real-time flow of mitogen-activated protein kinase across the NE was observed, demonstrating that its nucleocytoplasmic shuttling rate increased following growth factor stimulation.

Outlook

It is very intriguing to study the spatial regulation of a biological function within a sample at body, organ, cell, organelle and small domain levels. Whereas very few LM techniques for 3D reconstruction can penetrate tissue blocks thicker than 1 mm, most tomographic techniques, including optical projection tomography [50], fluorescent protein tomography [51], computed tomography and positron emission tomography, as well as magnetic resonance imaging can analyze structural and quantitative features in much larger tissues, such as the whole body. Although current tissue clearing techniques are limited to fixed biological samples, they are expected to enlarge the volume of 3D reconstruction from LM data, thereby bridging the imaging gap [52] between the size of a specimen that can be visualized with LM and that with other techniques.

Likewise, the imaging gap between LM and EM is also being buried by increasing the spatial resolution of fluorescence imaging (super-resolution techniques) [53–57] or by strengthening the interactions between LM and EM (correlative LM/EM techniques) [58,59], which are both reviewed elsewhere. We have high expectations regarding technological innovations for visualizing biological functions.

Funding

This work was partly supported by grants from Japan Ministry of Education, Culture, Sports, Science and Technology Grant-in Aid for Scientific Research on Priority Areas.

Acknowledgements

We thank Hiroshi Hama and Hiroko Sakurai for help with preparing this review.

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