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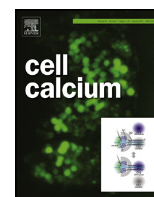

Methods for monitoring signaling molecules in cellular compartments

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ABSTRACT

Cells, irrespective of whether they are from multicellular or single-celled organisms, must communicate with the external environment through dynamic regulation of their internal metabolism, which are critical for their survival. Fluorescent and bioluminescent proteins, and related genetic engineering technologies, have provided new opportunities to investigate the molecular dynamics of cells and their internal compartments, with high spatio-temporal resolution. In this review article, since there is a sufficient number of previous reviews summarizing the history of their development and the techniques behind them, here we will focus on molecular features or technologies that have the potential to further open novel investigations of cellular and subcellular dynamics.

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1. Introduction

Cells, irrespective of whether they are from multicellular or single-celled organisms, must communicate with the external environment through dynamic regulation of their internal metabolism in order to survive and multiply [1,2]. To study the internal workings of the cell, fluorescent/bioluminescent proteins and related genetic engineering technologies have provided new opportunities to investigate the molecular dynamics of cells and their internal compartments, with high spatio-temporal resolution [2,3].

To monitor local phenomena in intact cells, probes must be non-invasively integrated into cells and cellular compartments, and thus conventional methods, including those based on synthetic chemical dyes, have some technical limitations. In contrast, protein-based probes are genetically encoded, and genetic engineering techniques allow us to “hijack” natural proteins [4], which enables 1) tagging of endogenous proteins to trace their mobility or to locate probes in a specific compartment, and 2) fusing of the sensing domains of natural proteins with fluorescent/bioluminescent proteins, as indicators to monitor molecular dynamics (e.g., developing calcium indicators). In the latter case, quantitative measurement can

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be performed based on changes in the brightness of the indicators [3,4].

To date, many fluorescent/bioluminescent proteins have been identified and developed, and a tremendous number of derived indicators are available, enabling precise observation of biological phenomena at subcellular resolution, with high temporal precision [5,6]. Since the history of their development and the underlying molecular engineering techniques have previously been summarized [3–6], here we will focus on molecular features or technologies that have the potential to further open novel investigations of cellular and subcellular dynamics.

2. Investigating molecular dynamics at subcellular resolution: tips to overcome difficulties with conventional imaging techniques

Both synthetic dyes and genetically encoded proteins are available for optical imaging in cellular compartments. Here, we will mainly focus on genetically encoded proteins, which can easily and simply be used to target cellular compartments and molecules. We suggest characteristics and features of genetically encoded proteins that we think are worth considering, especially when tackling questions that were previously difficult to address. After discussing techniques related to fluorescence imaging, we also discuss recent advances in bioluminescence-based imaging, which may help to solve several problems in fluorescence imaging.

2.1. Multi-color imaging

Development of multiple color variants of fluorescent proteins (FPs) and indicators (sensors) is crucial for monitoring of subcellular dynamics, for two main reasons: 1) it allows simultaneous monitoring of multiple molecules/components and their interactions, and 2) it permits development of indicators based on Förster (or fluorescence) resonance energy transfer (FRET) [7]. After green fluorescent protein (GFP) and its derivatives showed the potential of multi-color approaches [4,8–10], various new proteins were cloned and modified for "expansion in the palette of FPs" [4,11–13].

Recently, far-red and near-infrared fluorescent proteins have been reported [14–16], and the long emission wavelength variants have enabled more efficient detection of signal from deep and/or thick samples. This will likely be advantageous for *in vivo* imaging, and may permit investigation of subcellular dynamics. These near infrared proteins are based on bacterial phytochrome photoreceptors, which require biliverdin as the chromophore[14,15], indicating that their advantage (longer wavelength) and disadvantage (necessity of external chromophore) may need to be considered carefully when choosing FPs for each experimental condition.

These color variants have also been used to develop color variants of indicators [5,6,17–20]. Moreover, they allow various types of combinations, enabling a wide range of studies investigating the relationships between multiple molecules and cellular microcompartments.

In addition, FPs with a large Stokes shift, in which the emission peak wavelength is much longer than the excitation peak wavelength (90–180 nm) than usual (~50 nm), not only contribute to expanding the palette but also enable multicolor imaging with single-wavelength excitation [21,22]. The recently developed CyOFP1, a large-Stokes-shift FP, emits orange light with cyan light excitation, and is substantially brighter than previously-developed large-Stokes-shift FPs [22]. CyOFP1 was used for *in vivo* demonstration of the capability of single-wavelength, two-photon-excitation based, dual-color imaging combined with a green Ca^{2+} indicator, GCaMP6s, to monitor neural activity [22]. If a Ca^{2+} indicator based on CyOFP1 could be developed, it, as well as the previously developed large-Stokes-shift Ca^{2+} indicator REX-GECO1[23], would further enable efficient dual-color Ca^{2+} imaging in different cellular compartments or subtypes simultaneously.

2.2. pH sensitivity

pH is another important feature to consider when choosing an indicator to monitor subcellular events. The interior and exterior of different subcellular compartments tend to have different pH environments, ranging from 4.5–8; they can change dynamically depending on cellular activity and developmental status [24,25].

Many fluorescent proteins are somewhat pH sensitive [26]. This is a potentially disadvantageous feature for quantitative comparison of signals from different compartments. On the other hand, methods that utilize the pH sensitivity as a positive feature have been developed. pH-sensitive green FP–based sensors (pHluorins) have been used to monitor the exocytosis and endocytosis of synaptic vesicles [27]. Color variants have been reported, and demonstrated to be useful for multi-color pre- and post-synaptic imaging, as well as to show applicability to "all optical manipulation," a technique that combines optogenetic manipulation (which will be discussed later) and optical imaging [28].

There remains a demand to develop pH-insensitive probes, for quantitative comparison between different cellular compartments. The Sirius protein has been reported to be pH insensitive, and to retain sufficient brightness even in acidic environments, enabling continuous observation in phagosomes during phagocytosis [26]. Further improvement of these tools, or identification of novel ones, may encourage study of acidic cellular compartments.

2.3. Optimal affinity of indicators

When designing indicators to monitor molecular dynamics, affinity to the target compound is crucial. While a higher dynamic range is generally more advantageous to correctly and precisely detect actual changes in each environment, a higher affinity is not necessarily better, as it depends on the context. The ideal indicator is the one possessing a K_d value and Hill coefficient that covers the actual physiological concentration in the target environment.

For this purpose, optimization of the indicator for each target concentration is sometimes necessary, while it is convenient if there is a series of indicators of different affinities.

It has been suggested that presynaptic motoneuron boutons of *Drosophila melanogaster* larvae might have a very low cytosolic Ca^{2+} concentration, and show very small changes during action potential [29]. In this situation, high-affinity indicators, such as the YC-nano series [30], should be tested as the first candidates. In contrast, the Ca^{2+} concentration in the endoplasmic reticulum (ER) is assumed to be relatively high, reaching the sub-millimolar range [31]. To monitor the Ca^{2+} dynamics in the ER, low-affinity indicators such as CEPIA and its variants, including color variants, have been developed [31]. As shown in this work, combining multiple indicators optimized for different environments also allows imaging of intraorganellar Ca^{2+} signaling.

It should be noted that indicator kinetics is also a very important feature, especially when monitoring rapid phenomena, such as Ca^{2+} changes during neuronal action potentials [20]. Frequently, there is a considerable tradeoff between the kinetics and dynamic range of an indicator [5,6,20], and other conditions, including expression efficacy, may also vary depending on cell types and samples. Therefore, to identify the best indicator for each study, it may be advisable to test multiple candidates, if available, before deciding which one to use.

On the other hand, as for the tradeoff between kinetics and affinity, a recent study demonstrated that more rational designing can

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