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Subcellular functions of proteins under fluorescence single-cell microscopy[☆]

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Abstract

A cell is a highly organized, dynamic, and intricate biological entity orchestrated by a myriad of proteins and their self-assemblies. Because a protein's actions depend on its coordination in both space and time, our curiosity about protein functions has extended from the test tube into the intracellular space of the cell. Accordingly, modern technological developments and advances in enzymology have been geared towards analyzing protein functions within intact single cells. We discuss here how fluorescence single-cell microscopy has been employed to identify subcellular locations of proteins, detect reversible protein–protein interactions, and measure protein activity and kinetics in living cells. Considering that fluorescence single-cell microscopy has been only recently recognized as a primary technique in enzymology, its potentials and outcomes in studying intracellular protein functions are projected to be immensely useful and enlightening. We anticipate that this review would inspire many investigators to study their proteins of interest beyond the conventional boundary of specific disciplines. This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

Keywords

Fluorescence microscopy; Genetically-encoded fluorescent tag; Subcellular localization; Colocalization; Protein–protein interaction; Protein activity; Enzyme kinetics

1. Introduction

Enzymology, the study of proteins and their functions, has been a mainstream of biochemistry; not only before the pre-genomic revolution but also in the post-genomic era. To date, *in vitro* enzymology has made a tremendous contribution to our current understanding of a cell. However, the *in vitro* experimental conditions used to study proteins and their functions are only a rudimentary reflection of the proteins' native intracellular space. In order to truly appreciate the breadth of a protein's function, enzymology techniques have continually been modernized to interrogate the often transient and dynamic

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interactions inside a cell, which otherwise cannot be replicated *in vitro*. Accordingly, there have been tremendous efforts to correlate the *in vitro* function of a protein with the mechanism of its action in the heterogeneous nature of the cellular milieu.

In this review, fluorescence single-cell microscopy is emphasized as an essential tool for *in-cell* enzymology. Although fluorescence single-cell microscopy has been an established tool in cell biology, it has only recently become a promising technique for enzymology. Conventionally, fluorescence single-cell microscopy offers easy ways to monitor subcellular locations of proteins with great target-specificity and spatial resolution in single cells. Recent advances in quantitative *in-cell* Förster resonance energy transfer (FRET) microscopic techniques have enabled the real-time measurement of spatiotemporal protein–protein interactions inside cells. Consequently, cellular communication networks have been visualized between various cellular processes, including but not limited to, metabolism, signaling pathways, and organelle biogenesis. Furthermore, the development of novel molecular biosensors has allowed researchers to quantify kinase/phosphatase activities and their dynamics inside living cells.

However, it still has been challenging to precisely measure enzyme kinetics, the hallmark of enzymology, inside living cells. Considering that accurate measurements of enzyme kinetics rely on the interactions between the enzyme of interest, its substrate(s), and often a co-factor, their spatiotemporal concentrations cannot be readily assumed nor can a time zero be effortlessly controlled to initiate the measurement of enzyme kinetics. Therefore, we anticipate that this review discussing protein labeling strategies (Fig. 1) and biophysical microscopic techniques (Fig. 2) provides the foundational knowledge of future innovative ideas and/or interdisciplinary strategies for *in-cell* enzymology.

2. Labeling strategies for fluorescence single-cell microscopy

In order to study a protein under fluorescence single-cell microscopy, the protein must be labeled. This label can either be a genetically-encoded tag, which often requires a functionalized fluorophore, or a synthetic chemical fluorophore that is covalently conjugated *in vitro* to the protein or to the protein-specific antibody. The benefit of the former is that experimentation can be accomplished in live cells with minimal perturbation, while the latter requires micro-injection into live cells or chemical manipulation for cell fixation and permeabilization. We will focus on genetically-encoded tags here as they enable real-time measurement of protein activity inside live cells under fluorescence single-cell microscopy (Fig. 1). Nonetheless, a cohesive combination of various labeling methodologies is desired to unambiguously identify and corroborate novel spatial and temporal dynamics of proteins inside cells.

2.1. Fluorescent protein tags

The discovery of *Aequorea victoria*'s green fluorescent protein (GFP) and its subsequent analogs has revolutionized the study of proteins and their intracellular dynamics [1,2]. Researchers have isolated, characterized, and modified dozens of fluorescent proteins from jellyfish, coral, and anemone species to meet a variety of research needs [3]. The result is a repository of fluorescent proteins ranging in color, brightness, stability, and maturation time

[4]. Therefore, in order to promote innovative usages of fluorescent proteins in the field of *in-cell* enzymology, we recapitulate here the advantages and disadvantages of using fluorescent proteins inside living cells.

Fluorescent proteins have reigned supreme in fluorescence microscopic imaging for several reasons. First, the chromophore formed inside the β -barrel structure of a fluorescent protein allows long-term visualization of the tagged protein of interest in cells because of the chromophore's resistance to pH and temperature variations, as well as proteolysis in mammalian cells [1]. Second, background fluorescence and noise are significantly reduced due to the ensured molecular-level specificity with a 1:1 labeling ratio. Third, encoding a fluorescent protein-tagged protein minimally perturbs the cells compared to alternative protein labeling strategies employing *in vitro* fluorophore-conjugated proteins or antibodies.

Fluorescent proteins, however, have their fair share of experimental perversities [5,6]. Fluorescent proteins fused to the terminal ends of proteins may obstruct the purpose of the organelle-specific localization sequences that target proteins to their subcellular location [7]. Fluorescent proteins can also disrupt proper protein folding [8] and oligomerization [9], as well as cause steric hindrance disabling interactions with protein partners and subcellular structures [10]. Furthermore, fluorescent proteins themselves may form oligomers when fusion proteins are overexpressed or localized to spatially confined areas in cells, such as the endoplasmic reticulum [11,12] and the plasma membrane [6, 13]. Collectively, careful deliberations and considerations of fluorescent protein-tagged proteins are required to avoid aberrant localization patterns and misleading visual artifacts.

2.2. Small polypeptide tag

In search of a smaller, less obtrusive genetic tag, researchers have developed the genetic tag-probe labeling methodologies, which utilize biologically compatible or 'bioorthogonal' chemical reactions.

The tetracysteine motif is one such small polypeptide tag, whose amino acid sequence rarely occurs in nature. It contains the N -CCXXCC- C sequence, where C is cysteine and X is any amino acid, which becomes fluorescent only upon strong non-covalent binding to biarsenical compounds, such as the derivatives of fluorescein (e.g. FAsH) and resorufin (e.g. ReAsH) [14]. This method provides the benefit of multimodal imaging with a single tag, and on account of its size, is a valued alternative to labeling intracellular targets that have suspected or confirmed fluorescent protein-induced aberrations [15]. However, the biarsenical probes have been reported to not only cause cell stress, but bind non-specifically to cysteine-containing proteins [16]. The increased background signal presents a challenge in studying low-abundance proteins because iterative washing to reduce the background signal may potentially exacerbate cell stress. Furthermore, the biarsenical interaction requires a reducing environment and thus applied to study mainly cytosolic proteins [17].

Meanwhile, alternative strategies employing small polypeptide tags have been expanded at least for cell-surface proteins. The polyhistidine tagged surface proteins show reversible binding with multivalent nitrilotriacetate-conjugated fluorophores [18]. Similarly, a few bio-orthogonal strategies utilize an enzyme-mediated labeling process where either *Escherichia*

coli's biotin ligase, formylglycine generating enzyme, or phosphopantetheinyl transferase is used to covalently attach or generate a ketone/aldehyde-containing moiety within the fusion protein localized on the cell surface [19–21]. These peptide tags may be considered superior to their fluorescent protein counterparts as long as cell surface proteins are the primary targets of fluorescence imaging.

3. Investigating the subcellular location of proteins

The subcellular location of a protein has been commonly described as being cytosolic, membrane-bound, or internalized within conspicuous membrane-bound organelles. We are recently starting to learn, however, that these subcellular addresses are a drastic oversimplification of a higher spatial organizational hierarchy that exists within the compartmentalized phases of the cell [22,23]. Fluorescence single-cell microscopy has played an essential role in the identification of non-membrane bound cellular bodies [23–28], that localize reversibly in response to cellular needs and/or stress. Furthermore, the translocation of proteins within and between cellular compartments has been used to help identify novel functionalities (i.e. moonlighting) [29] and establish how spatial rearrangement of a protein can be employed by the cell to achieve its functional diversity in a myriad of cellular processes. Therefore, fluorescence time-lapse imaging and colocalization microscopy are discussed here as a means of exploring subcellular location-specific roles of a protein in the intracellular space.

3.1. Fluorescence time-lapse imaging

The most significant advantage of performing fluorescence time-lapse imaging in single, living cells is that the intracellular motions of a protein can be measured in real-time with a high spatial and temporal resolution. This is critical because intracellular events are naturally transient and dynamic phenomena. Fluorescence time-lapse imaging of a protein in single cells has not only revealed its subcellular locations in time and space [24,30], but also has great potential to unveil various stages of novel spatiotemporal dynamics of a protein within various subpopulations of cells, which are otherwise obscured in ensemble-averaged observation.

Importantly, time-lapse imaging has continuously evolved to make it more quantitative, robust, and/or employed in automated high-content screening assays [31]. In particular, time-dependent protein translocation has been successfully quantified as a relative ratio of fluorescence intensity between two user-defined regions of interest within a cell over a specified time-course. Intensity-based line or area analyses have quantified relative changes of fluorescent intensities between the plasma membrane and the cytoplasm [32–35], whereas quantification of protein translocation between the cytoplasm and the nucleus has been effectively employed with automation in high-content screening assays [36,37]. Therefore, these quantitative strategies have been very useful in investigating how the stimulus-instigated spatiotemporal rearrangement of proteins can affect subcellular location-specific functions of proteins inside a cell.

3.2. Colocalization microscopy

Colocalization microscopy with at least two protein targets has been employed to unveil novel and transient macromolecular organizations or compartments that would otherwise not have been reconstituted *in vitro*. One such application has been in the identification of a multiprotein complex involved in cholesterol transport to the mitochondria [38]. Briefly, steroids triggered translocation of a translocator protein-associated protein from the Golgi complex to mitochondria, promoting colocalization with other associated proteins to form a multiprotein complex involved in cholesterol transport. In another example, the six enzymes catalyzing human *de novo* purine biosynthesis undergo microtubule-assisted colocalization in the cytoplasm under purine-depleted conditions [24,39]. Along with the colocalization event, various biochemical and biophysical studies have supported the formation of a macromolecular metabolic compartment for *de novo* purine biosynthesis, termed the purinosome, in cancer cells [24,40]. Although colocalization of two or more proteins does not necessarily indicate their direct interactions, spatial clustering of a group of sequential metabolic enzymes has been shown to increase metabolic flux [39, 41] through cluster-mediated channeling [42]. Therefore, colocalization microscopy itself, even without measuring direct interaction between colocalized proteins, appears to aid in understanding the functional activity of multiprotein organizations.

4. Identifying cellular protein–protein interactions

The investigation of protein–protein interactions has been one of the central purposes of *in vitro* enzymology because it allows us to better understand the functional diversity of proteins and the role they play in the context of the cellular milieu. However, a number of established *in vitro* techniques are mostly limited to identifying stable, rather than transient, protein–protein interactions. Even, such protein–protein interactions are in some instances speculated not to occur in the cell the way *in vitro* studies predicted [43,44]. Therefore, studying both stable and transient protein–protein interactions within the cell is particularly important for enzymology. Here we discuss briefly the most commonly used biophysical techniques and how they have been applied to understand protein functions using fluorescence single-cell microscopy.

4.1. Förster resonance energy transfer (FRET)

Despite the power of colocalization studies to investigate the spatial and temporal localization of proteins within the cell, colocalization microscopy is diffraction limited to determine direct protein–protein interactions. Therefore, as in *in vitro* enzymology, FRET has also played an imperative role in quantitative measurement of direct protein–protein interactions inside cells.

Briefly, FRET describes the phenomenon that occurs when a donor fluorophore non-radiatively transfers energy to an acceptor fluorophore, emitting red-shifted fluorescence. The efficiency of this energy transfer between the donor–acceptor pair is inversely proportional to the distance between the two raised to the sixth power [45]. Consequently, FRET efficiency rapidly declines as the donor–acceptor pair separate and diminishes at distances greater than 10 nm. Because of this sensitivity to distance, FRET has been

successfully used inside cells as a molecular ruler to identify protein–protein interactions. Such examples include, but are not limited to, signaling pathways, protein oligomerization, and macromolecular complexes [46]. Therefore, *in-cell* FRET measurements will be the choice of researchers to explore the functional roles and interactions of proteins inside cells.

Several quantitative techniques measuring *in-cell* FRET signals have been developed for fluorescence microscopy [47]. First, the sensitized emission method measures the concomitant decrease in donor and increase in acceptor emission intensities as FRET is occurring. Due to the excitation and emission spectral overlaps between donor and acceptor fluorophores, this method requires obtaining a control set of images from cells expressing only the donor and only the acceptor in order to extract out *bona-fide* FRET signals from the specimen co-expressing both the donor and acceptor proteins. Second, the photobleaching method measures the fluorescent intensity changes in either donor or acceptor intensity following photobleaching of the other. For acceptor photobleaching, the donor's steady-state emission intensity is measured pre- and post-bleach of the acceptor, where an increase in donor signal is indication of FRET occurrence. For donor photobleaching, the rate of photobleaching of the donor is measured in both the absence and presence of the acceptor, where a decrease of the donor's photobleaching rate in the presence of acceptor indicates FRET. Third, fluorescence lifetime imaging microscopy measures the fluorescence lifetime of the donor, rather than fluorescence intensity, in the presence and absence of acceptor. The trade-offs for the above methods can be simplified as follows; those measuring FRET signals as changes in fluorescence intensity (i.e. sensitized emission and photobleaching methods) can be accomplished on commercialized confocal microscopes, but require considerable image processing using various controls for correction, while fluorescence lifetime imaging microscopy, where relative fluorescent intensities of donor and acceptor molecules have no impact on FRET efficiency, requires very expensive and custom-made instrumentation that is not readily available to many researchers [47].

In addition, FRET studies have advanced to measure protein–protein interactions between three target proteins inside living cells. First, three-chromophore FRET or 3-FRET was developed to utilize three fluorescent proteins to create two FRET pairs (e.g. cyan fluorescent protein, yellow fluorescent protein, and monomeric red fluorescent protein) [48]. Their 3-FRET efficiency was measured to demonstrate the interactions between membrane-bound receptor proteins in response to stimulus and, further, to determine if the three protein interactions are sequential or in parallel. Second, a sequential combination of bioluminescence resonance energy transfer and FRET was used to observe heteromerization of G-protein coupled receptors in live cells [49]. Here, a bioluminescent luciferase protein acts as a donor to a fluorescent protein, which then acts as a FRET donor to a third fluorescent protein. Considering that luciferase can have many substrates and thus various emission profiles [50], this method becomes compatible with several fluorescent proteins as downstream interaction reporters [49,51].

4.2. Bimolecular fluorescence complementation (BiFC)

In addition to FRET-based methods, bimolecular fluorescence complementation (BiFC) or protein complementation assays have been developed to study intracellular protein–protein

interactions. This method relies on complementation efficiency of two fusion proteins, each containing one half of a fluorescent protein. When the two target proteins interact, the fluorescent protein halves come together in proximity, forming a mature fluorescent protein. Since fluorescent signals themselves indicate direct observation of protein–protein interactions in the cell, this method does not require specialized instrumentation, thus making this technique versatile. Indeed, BiFC has been successfully employed to study target-specific protein–protein interactions [52–55] and further applied in high-throughput screening assays [56–58].

However, post-imaging analysis of the BiFC-mediated results should take into account the intrinsic properties of the fluorescent protein itself. Due to the required maturation time of the fluorescent protein in cells, transient protein–protein interactions may be missed by the delayed response of fluorescence signals from the protein–protein interaction [54]. In addition, the fluorescent protein fragments may self-associate and form a mature fluorescent protein without an interaction of the target proteins of interest, especially when overexpressed, thereby causing false-positive results [59,60].

5. Measuring protein activity through biosensors in cells

Studying a protein's activity and kinetics in their native environment is a very challenging, yet essential task. An enzyme's activity may greatly depend upon the subcellular location [61], where intracellular cues ripple through multi-protein signaling networks and also the concentrations of myriad metabolites and cofactors oscillate across the cell. To understand the subcellular location-specific activity of an enzyme, great strides have been made in the development of biosensors –genetically-encoded molecular switches – that are capable of measuring enzyme activity and kinetics in living cells [4]. These biosensors are indicative of the progress that has been made towards *in-cell* enzymology, effectively reshaping enzymology approaches towards elucidating and characterizing proteins and their functions inside cells.

5.1. Measuring protein kinase activity

In eukaryotic cells, a large family of enzymes, known as protein kinases, catalyze the transfer of a phosphate group to their protein substrates using ATP as a phosphate donor; i.e. phosphorylation. A series of kinase-mediated phosphorylation events often results in cascades of signal transduction in cells. The activity and dysregulation of protein kinases have been intimately tied to a number of human diseases, including, but not limited to, cancer and diabetes [62–65]. Typically, the activity of kinases is measured using either radiological analysis or western blots with antibodies specific for phosphorylated substrates. However, these methods do not provide information about how kinase activity is differentially sequestered temporally in a subcellular location-specific manner.

Accordingly, many of FRET-based kinase biosensors have been developed and have validated subcellular location-specific kinase activities inside living cells [66]. In principle, the genetically-encoded biosensor acts as a surrogate substrate for the target kinase, composed of both a kinase-specific substrate motif and a phospho-amino acid recognition domain labeled with a FRET pair. Upon phosphorylation, the phosphorylated substrate motif

binds to its recognition domain, bringing the two fluorophores in proximity for FRET to occur. This FRET signal is indication of protein kinase activity in living cells. For example, the activity of protein kinase A was measured in the plasma membrane upon both the formation of lipid membrane rafts and the addition of growth factors [67]. The spatiotemporal oscillation of calcium and cyclic AMP was further revealed to be regulated by the location-specific cellular activity of protein kinase A [67]. In addition, an AMP-activated protein kinase (AMPK) biosensor, composed of an AMPK substrate motif and a fork head associated domain 1 inserted between a pair of fluorescent proteins, was developed to study the oscillation of *in-cell* AMPK activity in response to various stimuli, such as calcium or energy stress [68].

In other instances, however, the surrogate substrate can be eliminated. If the kinase itself undergoes conformational change upon activation, donor–acceptor fluorophores can be attached to the kinase and, accordingly, FRET signals are measured upon their activation-associated conformational changes [4]. For instance, 3-phosphoinositide-dependent protein kinase 1 (PDK1) was found to be active when it is associated with lipid raft domains in the plasma membrane upon growth factor stimulation [69]. Similarly, autophosphorylation-mediated conformational change of calcium/calmodulin-dependent protein kinase II was exploited as a biosensor to measure its activity in live neurons [70].

However, a number of biosensors measuring protein kinase activities are validated only in proof-of-concept systems. It is important to understand how a specific biosensor of interest has been designed, developed and evaluated. First, since a family of protein kinases share fairly similar recognition motifs, the sequence of a substrate motif selected for the biosensor should be very specific for the intended kinase [71]. Second, steric constraints should be considered when designing the bio-sensor, as false-negative results may arise if the engineered substrate motif in study is too small to be recognized as a biosensor [72]. Lastly, biosensors, which mostly work on the principle of FRET, require very strict controls in order to evaluate kinase activities measured from cells.

5.2. Measuring Michaelis–Menten kinetics of a protein phosphatase

Understanding enzyme kinetics has been the hallmark of *in vitro* enzymology. A FRET-based biosensor has been applied to study the kinetics of the endoplasmic reticulum-localized protein tyrosine phosphatase-1B (PTP1B) in live cells (Fig. 3) [73]. Briefly, the PTP1B phosphatase was tagged with a green fluorescent protein as a FRET donor, while its substrate containing a phosphorylated tyrosine was linked to a lissamine rhodamine B fluorophore as a FRET acceptor. The phosphate group on the substrate was chemically protected by a UV sensitive moiety, such that the phosphatase reaction could not be initiated without UV-induced photolysis of the protecting group. When the cells containing both the GFP-tagged PTP1B phosphatase and the rhodamine-tagged phosphorylated substrate were irradiated with UV light, the UV-mediated deprotection step defined the ‘time zero’ point to begin the measurement of the phosphatase reaction in a time-dependent manner. Post-processing analysis of FRET images allowed calculating both the Michaelis–Menten kinetics (i.e. K_M) and the catalytic rate of the enzyme directly from cells. Therefore,

developments in *in-cell* kinetic assays, that either utilize this strategy or are inspired by it (Fig. 3), will bring great excitement into the field of *in-cell* enzymology.

6. Two is one and one is none: corroborating experiments necessary for cell imaging

A popular adage amongst the military and survivalists, “Two is one, and one is none” summarily describes the utmost importance of having redundancies in tool kits as a fail-safe response to unforeseen circumstance. Such a philosophy can aptly be applied to fluorescence single-cell microscopy. All observations, especially when investigating protein localization and protein–protein interactions in cells, must be independently verified by not one, but several methods to ensure that spatiotemporal dynamics are *bona fide* cellular phenomena and not the result of aforementioned specific method-associated artifacts. Especially, researchers should ensure that localization phenotypes are reproducible irrespective of the fluorescent tag of choice. To validate localization phenotypes of the tagged proteins in live cells, immunocytochemistry visualizing endogenous proteins in mostly fixed cell specimens will confirm the phenotype is in no way caused by the tag or expression level. The opposite should also be said, because immunostaining has been reported to produce artifacts as well, due to the chemical treatment for cell fixation and permeabilization [74]. In addition, novel protein–protein interactions may occur only inside cells in certain circumstances due to unidentified third factors. In this case, a cohesive combination of the *in-cell* strategies mentioned in this review may be sufficient to validate the novel interactions as they cannot be readily replicated outside the cells. However, due to the lack of experimental approaches measuring the functional activity of a protein inside live cells, fluorescence single-cell microscopy is strongly recommended to complement, not replace, traditional *in vitro* biochemical assays. Thus, innovative techniques or strategies measuring protein activity inside cells are on demand for the future of *in-cell* enzymology.

7. Prospective: complementary techniques for fluorescence single-cell microscopy

Fluorescence single-cell microscopy is an extremely valuable tool in identifying the temporal and spatial dimensions of endemic protein function. However, the technique has yet to accomplish the level of quantification and control that can be achieved in *in vitro* enzymology. Along with fluorescence single-cell microscopy, it is imperative to recognize potential complementary strategies that would support ‘functional’ perspectives of a protein or protein complex of interest (Table 1). Here, we briefly talk about the potentials of metabolic flux assays, mass spectrometry imaging, and nanobody technology.

7.1. Metabolic flux assays

Metabolic flux assays that either use conventional radioactivity assays or modern quantitative mass spectrometric analysis have been shown to be great complementary techniques for fluorescence single-cell microscopy in identifying the functional activity of a metabolic pathway. Cellular colocalization of the enzymes in human *de novo* purine biosynthesis visualized under fluorescence single-cell microscopy was successfully

correlated with quantitative measurements of both the rate of the metabolic pathway [39] and the steady-state levels of the metabolic intermediates of the pathway [41]. Consequently, these results strongly support a positive relationship between subcellular localization and metabolic activity of purine metabolism in live cells. Retrospectively, a vast amount of metabolic flux assays carried out in the twentieth century in various cell lines and also recent measurements of altered metabolic flux in human cancer cells [75–77] may indicate plausible subcellular compartmentalization of metabolic pathways or enzymes beyond the conventional boundary of the organelles. We anticipate that the widespread application of fluorescence single-cell microscopy in the field of metabolism would be beneficial to comprehend subcellular location-specific functions of metabolic enzymes and thus their pathways in the cell.

7.2. Mass spectrometry imaging

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been successfully employed to measure the quantitative amounts of metabolites in animal and patient-tissue samples [78, 79]. Given the demonstrated high sensitivity and selectivity for individual metabolites in mass spectrometry, we envision that mass spectrometry imaging may have great potential to measure quantitative levels of small molecules reflecting protein activities in cells, if the spatial resolution of the technique is improved to resolve subcellular levels. In this regard, nano-scale secondary-ion mass spectrometry, which can achieve higher spatial resolutions than MALDI [80], has been used in combination with fluorescence microscopy for subcellular imaging of isotopically labeled drugs in cancer cells [81].

7.3. Nanobody technology

A nanobody is the smallest, intact antigen-binding fragment derived from a functional immunoglobulin, approximately 15 kDa in size [82]. Unlike antibodies, nanobodies can be overexpressed in *E. coli* for *in vitro* studies, and can be transfected into mammalian cells for *in-cell* applications. Conformation-specific nanobodies, indicating active or inactive conformations of a protein, have been recently used to determine the active forms of protein complex structures in X-ray crystallography [83,84]. Concurrently, GFP-tagged nanobodies that only recognize active conformers of proteins were employed under fluorescence single-cell microscopy to track the activity of internalized endosomes inside live cells [85]. The development of conformation-specific nanobodies for various proteins will be of great benefit in determining whether the proteins of interest are active or inactive at specific subcellular locations in live cells.

8. Conclusions

Fluorescence single-cell microscopy is reviewed here to promote its practical applications in the field of *in-cell* enzymology. We have discussed the topics of protein labeling strategies for groundwork, biophysical microscopic techniques for novel discovery, and compatible downstream analyses for *bona-fide* validation. The unconventional applications of existing technologies have demonstrated how ingenuity alone has redefined fluorescence single-cell microscopy as an innovative tool in enzymology. For example, molecular biosensors measuring post-translational modifications as an indication of protein activities in eukaryotic

cells represent a great success story employing already existing FRET-based imaging technologies. Particularly, a singular success example measuring Michaelis–Menten kinetics in live cells [73] has shed light on at least one potential direction for the future of *in-cell* enzymology (Fig. 3).

However, it is important to recapitulate that the precise measurement of protein activity and function in live cells is still challenging because the number of chemical reactions monitored by such molecular biosensors is significantly smaller than the number of chemical reactions occurring in a cell. We anticipate that fluorescence single-cell microscopy will contribute significantly to understanding a complete portrait of endogenous protein function, activity, and kinetics in single cells. Collaborative efforts bringing several expertise together (e.g. enzymology, biophysics, single-cell biology, and statistics) may be key to extending the horizon of current *in vitro* enzymology into the intracellular space of the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Overview for fluorescence single-cell microscopy.

Fig. 2.

Fluorescence single-cell microscopy for *in-cell* enzymology. Conventional fluorescence microscopy and biophysical techniques have been revitalized by their novel application towards understanding native enzyme function within live cells. Beyond protein subcellular localization, fluorescence single-cell microscopy has also been successfully used to visualize spatiotemporal dynamics of cellular protein–protein interactions using FRET technology. Measuring protein activity – even kinetics – within living cells is now possible using FRET-based biosensors in combination with this versatile technique.

Fig. 3.

Enzyme kinetics in live cells. One potential direction for measuring Michaelis–Menten kinetics of enzymes in cells is depicted based on a successful experiment that measured enzyme kinetics of tyrosine phosphatase, PTP1B, in living cells [73]. A GFP-conjugated construct of the phosphatase is introduced to cells alongside a phosphorylated peptide substrate that is linked to a FRET pair. Initial binding of the phospho-peptide substrate is prevented due to a protecting group. However, irradiation with UV light defines initiation of catalysis (i.e. $t = 0$) for time-dependent kinetic measurement. *In-cell* FRET signals reflect the catalytic activity of the phosphatase over time. These data can then be used to construct a Michaelis–Menten plot from which the catalytic rate and K_M can be derived. Note: Adopted beyond the reference [73].

Table 1

Overview of techniques complementary to fluorescence single-cell microscopy for a comprehensive characterization of intracellular protein function.

Technique	Advantages	Challenges	References
<i>Metabolic flux assays</i>	Numerous applications using various specimens, ranging from bacteria to mammalian cell culture Capable of analyzing metabolic fluxes in both isotopic steady-state and non-steady state	Insufficient knowledge about kinetic parameters of intracellular metabolic pathways Difficult to measure compartment-specific fluxes in eukaryotic cells	Extensively reviewed in [86,87]
<i>Mass spectrometry imaging</i>	Capable of quantifying heterogeneous distributions of small molecules with high sensitivity in tissue samples Label-free technique	Matrix-dependent extraction of small-molecule analytes from tissues No quantitative measurement of proteins from biological specimen Insufficient spatial and temporal resolution	Extensively reviewed in [78,88]
<i>Nanobody technology</i>	Robust recombinant expression in living cells with high specificity Distinguishable between active and inactive conformation of proteins in live cells	Potential steric hindrances and off-target effects in protein–protein interactions. Insufficient knowledge of its cellular and biochemical properties	Extensively reviewed in [89,90]