Challenges and recent advances in live cell bioimaging

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Live cell bioimaging is becoming an increasingly popular tool for elucidation of biological mechanisms and is instrumental in unravelling the dynamics and functions of many cellular processes. The imaging of live cells poses many challenges, such as cell viability, complex microscope settings and the use of appropriate fluorescent components that vary in nature. It is essential for a user to ensure live cell bioimaging is performed as near to the physiological states as possible in order to avoid occurrence of artefactual cellular behaviour. This article addresses the problems of phototoxicity, photobleaching and technical aberrations in time-lapse confocal and total internal reflection fluorescence microscopy and serves to provide the necessary solutions. The challenges of trouble-shooting the imaging of cellular endocytosis are illustrated as examples. In addition, the article discusses the applications and experiences in the use of new fluorescent dyes, baculovirus-based delivery of fluorescent proteins and biosensors in the study of a cellular endocytic event.

Keywords Time-lapse microscopy, TIRF, total internal reflection fluorescence microscopy, endocytosis, live cell imaging

1. Introduction

This chapter will elucidate the technical procedures in live cell imaging of a cell penetrating peptide that was carried out in Singapore Bioimaging Consortium-Nikon imaging (SBIC-Nikon) facility and update the readers on the latest commercial tools available that will contribute to the knowledge of live cell imaging techniques in deciphering of biological mechanisms. The SBIC-Nikon Imaging Centre provides technical assistance for confocal (A1Rsi microscope with simultaneous photo-bleaching and imaging abilities as well as spectral imaging) and motorized laser TIRF microscopy system (for multiple wavelength TIRF imaging).

![Bioimaging equipment at SBIC-Nikon imaging centre. (A) Nikon A1Rsi confocal system with spectral ability, encased in an OkoLab incubator and CO₂ regulator system (B) Nikon TIRF fluorescent microscope. (C) Glass coverslip holding chamber.](image)

Using the study of endocytosis of oligoarginine, a cell penetrating peptide, this article illustrates the various applications of confocal and TIRF imaging in the study of cellular entry and trafficking. Additionally, the problems encountered during acquisition of live cells will be discussed together with their respective solutions and considerations.

2. Glassware and imaging chamber

Currently, there are a vast array of slides, tissue culture dishes and cover slips of different formats that are commercially available for live cell imaging. Generally, the glass component should be 0.17 mm in thickness and have a numerical aperture (N.A.) close to 1.5. Various factors such as the cost, nature of the experiment and its compatibility to microscope system must be taken into consideration when selecting the specific glassware for the bioimaging experiments. Glass-bottomed dishes are typically more expensive than cover slips but can be handled more easily and without unnecessary exposure to the environment. This feature is especially crucial should the experiment involve an infectious agent or long-term imaging (>20 hours). It is also important to note that the size of the glass component varies across brands. Although selecting a glass dish with a wider surface area will provide more area for imaging, it...
will pose a challenge in locating the target cells if the cell density is too low. A glass dish with a smaller surface area is
often preferred when the imaging experiment involves embryos or eggs as it aids the user in localizing the target.

Alternatively, glass cover slips can be used as they are more cost-effective for short term imaging (< 8 hours) and
this also allows more duplicates of samples to be prepared for imaging. Despite the technical specification provided by
the manufacturers, compatibility of the glassware and microscopic system must be tested to avoid possible optical
problems. This is particularly critical when an automated focus system is used for live cell imaging. Another factor to
consider when choosing glassware for imaging is the nature of the cells of interest. Some cells (e.g. 293 HEK) which
detach easily will require glassware to be specially coated with poly-d-lysine or collagen to facilitate handling and
movement during imaging.

The imaging chamber or holder also comes in various configurations and specifications. Some models permit the
infusion of CO\textsubscript{2} and monitoring of temperature through an inbuilt thermostat while others function as perfusion
chambers whereby media containing compounds of interest can be introduced. In addition, microinjectors can be
adapted to fit the relevant perfusion chamber for imaging experiments involving micro-injection. An example of a cell
chamber is shown in Fig. 1C. This model from Nikon is engineered to enclose a 25 mm glass cover slip and is the
imaging chamber of choice for used all the experiments described in this chapter.

Other similar chambers are available commercially (e.g. Attofluor from Invitrogen). For a more detailed review of
the structural components and formats of other types of imaging chambers, please refer to a website cited under
reference [1]. The key consideration to be noted is ensuring compatibility between the imaging chamber and the
selected microscopic platform, stage and glassware.

3. Medium, pH and temperature control

The successful imaging of biological events in cells is largely dependent on the environmental conditions provided.
Ideally, the conditions should be as close to natural cellular ambience as possible in order to avoid the induction of
cellular stress responses. Hence, the medium, pH and temperature must be maintained at a physiological level that does
not alter biological processes of interest. The medium of choice is usually limited by the cell type of interest. In general,
the medium should meet all the nutritional requirements for cell growth and this usually involves the addition of serum.
Examples of popular media used in bioimaging are Dulbecco's modification of MEM (DMEM) and Roswell Park
Memorial Institute (RPMI). Serum-free media are also available but they should only be used for cells that are adapted
for serum-free environment (e.g. Freestyle CHO cells).

Most mammalian cell lines used in live cell imaging grow between pH 7.2 to 7.4. For bicarbonate-based media, the
buffering capability requires the presence of CO\textsubscript{2} and the sudden withdrawal of CO\textsubscript{2} is known to be detrimental to cell
growth [2]. Thus, the addition of buffering agents such as HEPES is important to minimize pH changes. However, the
presence of HEPES may not confer protection to the cells if they are exposed to the environment for prolonged periods
of time. Moreover, cellular toxicity of HEPES was also reported in some studies [3]. Therefore, monitoring the
sensitivity of the biological system to pH changes is absolutely crucial during initial testing. It is also observed that
DMEM is found to be more prone to pH changes than RPMI medium when incubated at room temperature. Although
the mechanism involved is unknown, one should choose the medium that offers the best cellular conditions to the cells
of interest. In situation where CO\textsubscript{2} supply is not available, one can consider the use of Leibovitz L-15 (L15) medium
which offers buffering protection through the use of amino acids and sodium pyruvate. However, the use of this
medium is restricted to few adapted cell lines (C6/36 mosquito cells). Although it is most recommended to use phenol
red-free media to prevent possible phototoxicity and interference of the fluorescence induced by the pH indicator, media
containing phenol red can be helpful in monitoring changes in pH during the imaging process.

Temperature is another key parameter to consider before one embarks on imaging experiments involving living cells.
Live cell imaging should be performed at the physiological temperature and this is usually achieved by using a
temperature regulatory system as depicted in Fig.1A, the Nikon microscope is encased in the OkoLab incubator coupled
with a CO\textsubscript{2} supply system. The set-up contains a thermostat that strictly regulates the temperature of the experimental
environment. The sensitivity to temperature fluctuations varies across cell lines, some cell lines such as BHK cells are
tolerant of ambient temperature while others like 293FT HEK and A172 (neuroglioblastoma) cells undergo cellular
detachment or changes in morphology upon prolonged exposure to room temperature. Thus, the maintenance of a
constant temperature is critical to preserve the viability of the cells. It is also important to mention the need to monitor
the volume of the medium when the temperature used is 37°C or higher as cellular perturbation caused by rapid
evaporation of the medium can occur during prolonged imaging process.
4. Phototoxicity and Photobleaching (laser & acquisition speed)

Fig. 2. Morphological changes in BHK cells due to phototoxicity such as (A) spindle-shaped membranes, (B) vacuolation and (C) rounding of cells. As shown in (D), the BHK cells displayed normal cell shape before imaging but prolonged exposure to activation laser caused membrane blebbing (indicated by arrow) and cell death (E).

Two most common challenges of live cell imaging are phototoxicity and photobleaching. Phototoxicity is the scenario whereby the cells undergo detrimental morphological changes such as the formation of spindle-shaped membrane structures (Fig. 2A), vacuolation (Fig. 2B), cell death (Fig. 2C) and blebbing (Fig. 2E) during the acquisition process. Such cellular damage can be induced by the rise in temperature caused by laser excitation or formation of free radicals during the excitation phase. This is largely attributed to the intensity of the laser and frequency of acquisition. High laser power can trigger heat stress responses in cells and lead to cell death. Similarly, the use of UV laser could also stimulate the production of reactive radicals that attack cellular membranes and lipids. Therefore, it is important to maintain the laser intensity at a minimum level in order to avoid extreme changes to the temperature of the medium and to reduce the occurrence of oxidative stress. In this study, we observed minimal phototoxicity and photobleaching when laser power applied is less than 10% for 488 nm laser and 10-15% for 561 nm laser were used respectively. Moreover, higher frequency of acquisition or use of multiple excitations can also contribute to phototoxicity. It is best to avoid the use of dyes that require excitation by the UV range (e.g. Hoechst) as they tend to stimulate phototoxicity in living cells. Other possible factors such as intrinsic sensitivity of cells to heat, radical damage and fluorescent dyes should also be taken into consideration when optimizing conditions for bioimaging. Addition of ascorbic acid or other antioxidants to the imaging medium may be helpful in reducing the level of free radicals [4]. However, this should only be attempted when the mechanism of interest is not affected by the antioxidant supplements.

Fig. 3. Effect of photobleaching on cellular fluorescence. Loss of fluorescence of FITC-labelled oligoarginine before (A) and after (B) exposure to strong laser power (indicated by arrows).

Photobleaching is the loss of fluorescent signals over time during live cell imaging (Fig. 3). The problem of photobleaching can be multifaceted. Apart from laser power and acquisition frequency, factors such as the photosensitivity of fluorescent dyes, the expression level of fluorescent proteins and the size of imaging objects are potential contributors to photobleaching. Photostable fluorophores (e.g. Quantum dots) are often preferred over organic dyes (e.g. TRITC) that are prone to the loss of fluorescent signals. The durability of the fluorescence may also significantly decrease if the expression level of fluorescent proteins is too low. Likewise, live cell imaging of small fluorescent vesicles is more susceptible to photobleaching compared to the image acquisition of larger fluorescent organelles (e.g. nucleus). Therefore, the application of laser power and acquisition frequency must be performed accordingly after considering the aforementioned factors in order to achieve the optimized settings required for the imaging of specific fluorescent objects. However, the optimization process can be frustrating when the setting for one fluorescent signal is suboptimal for another fluorescent dye used in the experiment. Careful selection of fluorescent dyes or proteins is thus critical for a successful imaging outcome.

4. Focal drift

A major challenge in live cell imaging is the axial fluctuations caused by gradual changes to the focusing of the sample during time-lapse experiment. This phenomenon is termed as focus drift and it defines the inability of the microscopic system to maintain the chosen focal plane over a period of time. Many factors contribute to the problem of focal drift, such as thermal drift, vibrations, addition of reagents and the movement of cells. Temperature variations caused by the ambient environment, intense laser intensity and the inefficiency of heating systems can caused focal drift. In addition,
vibrations generated by microscopic instruments, handling and environmental disturbances can also lead to the occurrence of focal drift. Although this can be eliminated by the installation of an anti-vibration table and by frequently securing the microscopic components, focal drift caused by the addition of reagents by hands remains dependent on the user. Two strategies are the use a narrow-tipped Pasteur pipette for chemical injection to minimize movement and the incorporation of a perfusion system onto the stage. An unavoidable problem in live cell bioimaging is focal drift induced by the motility of the cells (Fig. 4). Depending on cell types, living cells move during imaging and very often, the cells may shift beyond the focal plane or view. To circumvent this hindrance, one can try to restrict cellular movement by coating the culture dish with poly-l-lysine or fibronectin. However, this may also alter cellular behaviour.

To overcome the problem of focal drift, Nikon has developed a unique hardware based real-time focus correction solution, termed as the Perfect Focus System (PFS). PFS is designed to combat axial focus fluctuations that occur during long term time lapse imaging. The PFS system allows ultra fast sampling rate of 5 millisecond (200 Hz), which is independent of microscope and camera control software. It is much faster than other systems that require repetitive probing of cover slip interface and the focal plane of interest.

![Fig. 4](image_url)

**Fig. 4.** Images demonstrating the occurrence of focal drift during time lapse imaging. (A-C) The black shadows indicate the BHK cells and the green fluorescence indicates the oligoarginine in the medium. The change in cell shapes was due to focal drift that occur at the same optical plane over time.

### 5. Imaging an endocytic event

In this chapter, the study of the process of oligoarginine endocytosis was performed using time lapse confocal microscopy and TIRF fluorescent imaging. The main problem one will encounter during the imaging of endocytic events is finding a method or solution to differentiate the extracellular dye or labelled biomolecule from unstained cells. The use of widefield epifluorescence microscope to image endocytic event is strongly discouraged as it excites the fluorescence present in the medium at all optical planes and thus, the resulting fluorescence will completely obscure the visualization of the cells. The solution is to leverage on the ability of the confocal microscope to excite the laser at a specific optical plane, thereby separating the fluorescence present in the medium from the non-fluorescent cells. Similarly, the capability of evanescent waves to excite fluorescence at the membrane surface (~ up to 200 nm) can be manipulated to differentiate between the plasma membrane and the extracellular fluorescent biomolecules.

#### 5.1 Time-lapse confocal microscopy

There are two approaches to image the entry of fluorescent biomolecules. The first approach is to utilise the ability of the confocal microscope to dissect the cells into specific optical plane where the unstained cells can be clearly demarcated from the fluorescent extracellular environment. Although the disadvantage of this approach is the difficulty of achieving the desired optical plane without a reference dye, it provides an ideal imaging environment to track the entry of small fluorescent molecules if one manages to optimize the signal to noise (S/N) ratio. Rationally, the use of another dye (e.g. membrane dye) will facilitate the adjustment of the optical plane but the optical plane achieved by one dye may not necessarily correlate to the image plane of another dye. An alternative solution is the use of the Z-stack acquisition function so that one will reduce the likelihood of filming the entry processes at the wrong optical plane. However, this will also reduce the speed of acquisition. If imaging at different cellular localities is desired, the inclusion of the Z-stack function can further reduce the speed of acquisition. This could also pose a hindrance to the imaging of rapid cellular events that occur within the speed of seconds or less. Nonetheless, this problem can be overcome when a high speed confocal platform such as high speed resonant scanning coupled with piezo-z stage is used. In this study, we adopted the first approach with the inclusion of Z-stack function. Fig. 5 showed the time-lapse confocal imaging of the entry of FITC-labelled oligoarginine into BHK cells at a single optical plane.
Fig. 5. Time-lapse confocal microscopy of the entry process of FITC-labelled oligoarginine in BHK cells. Microscopy was performed on an inverted A1Rsi confocal microscope (Nikon) using a Plan-Apochromat 100× 1.4 N.A. lens. The cells were grown on 25 mm glass cover slips mounted onto holder (Fig. 1C). For detection of fluorescein-labelled peptides, the 488-nm line of an argon ion laser was directed over an HFT UV/488 beam splitter, and fluorescence was detected with a BP 505–550 band pass filter. Live cell bioimaging was performed at 37°C in 5% CO₂ microscope cage incubator system (OkoLab). The images were captured at 30 secs intervals at 1 frame per second (fps) for 30 mins and analyzed by NIS elements C software (64 bit, version 3, SP7/build 547). The black regions represent the cells and the green regions represent the medium containing the fluorescent peptides.

5.2 Fluorescence Recovery after Photobleaching (FRAP)

FRAP (Fluorescence Recovery after Photobleaching) is a microscopic technique where the fluorescent-labelled biomolecules in a demarcated area of the cell are irreversibly photobleached using high laser power. This is followed by the imaging of the subsequent trafficking of unaffected fluorescent molecules from the surroundings into the photobleached area. This method is effective for studying the dynamics and movement of biomolecules within the cells [5].

Two main parameters of concern in FRAP imaging are the laser power for photobleaching and the duration of photobleaching. If sub-optimal parameters for laser intensity and exposure timing are used, incomplete bleaching of the targeted fluorophores in the selected cellular compartment will occur. This is likely to affect the outcome of the quantitative data. Conversely, excessive laser power and prolonged duration of photobleaching might result in phototoxicity and off-target effects. As depicted in Fig. 6A-C, high laser power can cause membrane damages to the cell and allow the FITC-labelled peptides from the extracellular spaces to enter the compromised membranes. This might be misinterpreted as the recovery of fluorescence if the data is evaluated solely on quantitative results. Closer examination revealed that there is a distinct difference in cellular distribution of fluorescent peptides between the original sample (Fig 6A) and the “recovery” sample (Fig. 6C), thereby indicating possible membrane leakage. To avoid this problem, one should analyze the image while compiling quantitative information from the experiments. A successful FRAP experiment is demonstrated by Fig. 6D where re-entry of fluorescence molecules into photobleached region were imaged and quantified (Fig. 6E). The images at the region of interest were acquired at 5 secs interval for 2 mins before subjecting to photo-bleaching for 23.37 secs using 45% 488 nm laser intensity at 24 fps. This was followed by image acquisition of the region at 1 fps for every 10 secs for 1 hr using a Plan-Apochromat 100× 1.4 N.A. lens on A1Rsi confocal microscope. Image data were analysed using NIS elements C software to generate relative molecule diffusion rate.
5.3 Total Internal Reflection Fluorescence Microscopy (TIRF)

Total Internal Reflection Fluorescence (TIRF) microscopy is a method that selectively illuminates at the interface of contact between solids and liquids. It can specifically excite fluorophores in a cellular or aqueous environment that is in close proximity to a solid surface (~100nm), which generates an evanescent electromagnetic field that create images with very low background and out-of-focus fluorescence. Due to the remarkable signal to noise ratio and nano-scale membrane sectioning, TIRF imaging is widely used to image biological events occurring at the plasma membranes [6].

TIRF is a valuable tool for studying cellular endocytosis and exocytosis [7, 8]. This study employed the ability of TIRF to segregate the fluorescence detected from the plasma membrane from that of the medium to examine the entry process of oligoarginine in BHK cells. Before embarking on TIRF experiments, one needs to determine the method for visualizing cells when the sample is in the TIRF environment. In some exocytic studies, fluorescence vesicles fusing to the plasma membranes can serve as indicators for the focussing and adjustment of exposure timing. However, this is more challenging in the bioimaging of endocytic events as no reference points can be derived from the non-fluorescent cells at the start of the experiment for optical adjustment. To overcome this problem, a post-entry fluorescent sample can be prepared for optimization of microscopic parameters before the commencement of the actual experiment. This approach was used to derive Fig. 7. Further improvement can be performed by using another fluorophore to visualize the plasma membrane.

For the labelling of plasma membranes for TIRF imaging, organic fluorescent membrane dyes are not suitable as they bind non-specifically to the cover slip surface. Thus, preliminary study should be done to find a suitable labelling agent. An excellent alternative is to express RFP targeted to the plasma membrane using the Myristoylation/palmitoylation sequence from Lck tyrosine kinase (Organelle Light-Plasma Membrane, Invitrogen). This offers simultaneous imaging of entry of FITC-labelled oligoarginine and plasma membrane. Another factor that affects TIRF imaging is the confluency of cells as high cell density can interfere with the distinguishing of individual cells during imaging.

Fig. 6. (A-C) Artefactual recovery of fluorescence due to phototoxicity. Images depict the sequential events whereby the target cell was subjected to a short phase of photobleaching (A), followed by the image acquisition for fluorescence recovery (B) and the re-entry of FITC-labelled oligoarginine due to compromised membranes (C). (D) The recovery of fluorescence molecules after photobleaching using optimized settings and the trend line of fluorescence recovery is quantified (E). Red circles indicate regions of interest while green circles indicate region selected for background control.
Fig. 7. (A-D) The entry process of FITC-labelled oligoarginine in BHK cells using time lapse TIRF imaging. A motorised TIRF illumination system, based on a Nikon eclipse Ti-E inverted microscope with an Apo TIRF 100×NA 1.49 lens at 37°C/5% CO\textsubscript{2} TIZ stage-top incubator system (Tokai Hit) was used. The TIRF 488 nm excitation laser was used to observe the cell entry process of oligoarginine in BHK cells. Live cell imaging was acquired by a photometric CoolSnap HQ camera in a 2 X 2 binning mode at 100 ms exposure at 10 MHz readout speed. Image analysis was performed with NIS Elements AR 3.1 software (Nikon). Gradual increase in green fluorescence signified the entry of oligoarginine into the plasma membrane over time.

6. New tools in the market

In recent years, scientists have witnessed the advent of super-resolution imaging hardware such as STED and SPIM as well as the development of various fluorescent tools. The following texts focus on some of the new fluorescent dyes, proteins and biosensors that are available commercially.

6.1 New Fluorescent dyes

The Phrodo dye (Invitrogen) is a proprietary pH-sensitive dye that can be used to study endocytic events. The dye is essentially non-fluorescent at neutral pH but emits a strong red fluorescence upon exposure to an acidic environment. The increase in fluorescence signals correlates to the decrease in pH value. Thus, this property can be manipulated to investigate the effect of drugs or environmental parameters on cellular pH. Moreover, the dye emits minimal fluorescence in the medium thereby eliminating washing or quenching steps during processing. Currently, this dye has been used successfully to label bacteria [9], viruses (unpublished data) and dextran for the study of endocytosis and autophagy [10]. The pH-sensitive function of Phrodo was harnessed in this study for measuring the effect of oligoarginine on vesicular pH. As shown in Fig. 8A, BHK cells pre-treated with Phrodo-conjugated dextran displayed strong vesicular fluorescence before exposure to oligoarginine. Initial time-lapse acquisition of cells was performed for 3 to 5 mins to derive the pre-treatment fluorescence values before addition of the peptides. Subsequent introduction of oligoarginine caused reduction of Phrodo fluorescence in a time-dependent manner as indicated in Fig. 8B. The trend of pH changes can be analyzed quantitatively by selecting multiple cell targets (indicated by red, green and blue) and background control (yellow box) [Fig. 8C]. This experiment can be further improved by the incorporation of another reference stain to exclude fluorescence loss caused by photobleaching (e.g. Alexa Fluor 594- Wheat germ agglutinin).

Fig. 8. Measurement of pH changes in cellular vesicles using pH-sensitive Phrodo-dextran. (A) Cells displayed a bright red fluorescence before treatment with oligoarginine. (B) Reduction in Phrodo fluorescence after exposure to 10 micromolar of oligoarginine. (C) Fluorescence chart of pH values of oligoarginine-treated BHK cells over time constructed using NIS Elements software (Nikon).
For the labelling of cellular membranes, a number of new dyes were also introduced. These include red-fluorescent CellTrace BODIPY TR methyl ester (Invitrogen) that selectively stains endomembranous organelles such as the endoplasmic reticulum and the Golgi apparatus in live cells. It is useful for the studying of intracellular membranes [11]. Another dye is Wheat germ agglutinin protein conjugated to various Alexa Fluor dyes. Wheat germ agglutinin has a strong affinity for N-acetylglucosamine and sialic acid residues [12], which specifically stains the plasma membranes with low background noise. However, internalization of Wheat germ agglutinin-based membrane dyes can be a problem if imaging takes place for more than few hours. Alternatively, one can try CellMask Deep Red membrane amphipathic stain (Invitrogen) which supposedly exhibits a slower rate of internalization than Wheat germ agglutinin. It is important to note that Wheat germ agglutinin and CellMask dyes are not suitable for TIRF imaging as the dyes tend to stain the glass cover slips. Addition of BSA or repeated rinsing steps may help to reduce background noise.

Traditionally, the use of Hoechst dye in live cell imaging is strongly discouraged as the dye may cause cytotoxicity due to UV activation. However the toxic effects can be minimized by using a fluorescent dye like the Draq5 dye that does not require UV excitation. The Draq5 (Biostatus) stain emits strong fluorescence in the far red spectra and is compatible with other fluorescent components such as FITC. Furthermore, the use of Draq5 also means that another two cellular components can be imaged concurrently in the green and red spectra.

### 6.2 Photo-activatable proteins

A major leap in the cellular imaging is the introduction of photo-activatable fluorescent proteins, which can specifically tag the proteins of interest within a living cell. Wild-type GFP exists in two forms, which give rise to a major and a minor absorbance peak at 397nm and 475nm, respectively. Intense illumination at 400 nm shifts the population to the 475nm form, thereby increasing the absorbance of the minor peak. This capacity for photoconversion led to the development of PA-GFP. By selecting a form of GFP with a negligible 475nm peak, photoconversion produces a much greater proportional increase in 475nm absorbance compared with standard GFP and therefore increases contrast. PA-GFP exhibits very low green emission (max 517nm) after 488nm excitation and can be activated 100-fold by stimulation with 405nm light [13, 14].

PA-GFP is useful for studying temporal and spatial dynamics of proteins in living cells or tracking individual cells in a population of cells or in tissue. After activation, a clear increase in fluorescent intensity at the site of activation can be observed and the redistribution of activated molecules can be followed over time using time-lapse imaging (Fig. 9). This provides an alternative, and more direct, method, of molecular tracking compared to, for example, Fluorescent Recovery after Photobleaching (FRAP).

![Fig. 9](image_url) Measurement of protein dynamics using the Photo-activatable GFP in BHK cells. (A) BHK cells expressing mCherry (red) and PA-GFP (no fluorescence). (B) 488 nm laser excitation triggered photo-activation of PA-GFP (green). (C) Movement of PA-GFP from region of activation (green box) to region of detection (blue circle). (D) Chart depicts the increase in PA-GFP signal (green line) upon photo-activation at region of activation (green box) and subsequent decrease in signal as the protein trafficked to region of detection (blue circle). Blue line indicates the gradual increase in PA-GFP signal at region of detection (blue circle) as the photo-activated proteins moved into the region of interest.
6.3 Baculovirus-based delivery of fluorescent markers

The recent launch of the Organelle and Cellular Lights (Invitrogen) system helps the scientists to bypass the tedious route of cloning and generating stable cell lines by providing pre-packaged baculoviruses expressing fluorescent organelles or cellular markers. Baculovirus transduction is highly efficient in most cells and are apt for short term imaging (<72 hours). The key advantage of the new baculovirus system is the amenability of the technology to multiplexing with other organic fluorescent dyes, fluorescent proteins or quantum dots. This function is clearly demonstrated in Fig. 10, in which Organelle Light Golgi-transfected cells were prestained with ER tracker for concurrent live cell localization of oligoarginine in ER and Golgi apparatus. Another novel approach of multiplexing was to detect pH changes in lysosomes or endosomes in real time by combining the pH sensitive Phrodo-dextran and Organelle Light. This method increases the specificity of vesicular pH measurement by detecting changes only within lysosomes or endosomes (Figure 11). In addition, the Organelle and Cellular Lights are also compatible with normal fixation and immuno-staining protocols. However, one must note that the expression level of the fluorescent markers may vary across different cell lines and thus, the choice of cell lines must be evaluated carefully. The use of different fluorescent constructs might also influence the expression level and since a virus-based delivery is utilised, the potential interference on cellular responses should be evaluated.

![Fig. 10](image1)

**Fig. 10** Multiplexing of ER-Tracker Blue-White organic dye (A), FITC-labelled oligoarginine (B) and Organelle Light-Golgi (N-acetylgalactosaminyltransferase 2-RFP)[C] in BHK cells. Compatibility of Organelle/ Cellular Light systems with other fluorescent products facilitates live cell monitoring of multiple cellular components in a single setting. (D) Merged image with DIC.

![Fig. 11](image2)

**Fig. 11.** (A) Real-time detection of pH fluctuation within endosomes (Rab5a-GFP) by preloading of cellular vesicles with pH-sensitive Phrodo-dextran (red). (B) Magnified view of Phrodo-dextran within an endosome.

6.4 Fluorescent-based biosensors

The integration of baculovirus-mediated delivery and ion-sensitive fluorescent proteins also created a new line of biosensors for imaging. This range of sensors exploits the ability of various proteins that interact or translocate to different cellular compartments upon exposure to agonist or antagonists. The first product was the Premo™ Cameleon Calcium Sensor (Invitrogen) which permits the quantitation of calcium fluxes in living cells. The principle behind this technology is based on the ability of calcium ions to bind to calmodulin proteins and this in turn induces a conformational change that brings CFP and YFP domains in close proximity for forster resonance energy transfer (FRET) to occur. As shown in Fig. 12A-C, the addition of ATP, an agonist of calcium signaling pathway triggered the mobilization of intracellular calcium ions and induced FRET response. Such a response can be recorded in live cell imaging and used for quantification (Fig. 12D). Other commercially available biosensors include Premo Fucci that detect changes in cell cycle, Premo Halide that senses fluxes in halide ions and the LC3 biosensor that helps to identify the induction of autophagy.
Fig. 12. Detection of FRET in BHK cells transfected with Premo Chameleon. (A) Yellow fluorescence represents the expression of ECFP (green) and Venus YFP (red) at equilibrium before addition of calcium agonist, ATP. (B) Addition of ATP activated FRET reaction causing a reduction in fluorescence signal of ECFP and corresponding increment in YFP signal. (C) Figure depicts the return of ECFP and YFP fluorescence to equilibrium after cessation of calcium fluxes. (D) Chart depicts the dynamic fluctuations in FRET before and after the addition of ATP. Red line represents fluorescence signal of Venus YFP and green line represents fluorescence of ECFP.

7. Summary

The recent advancement of optical systems and fluorescent tools spur the renewed interest in live cell imaging. Imaging the cellular events in living cells and organisms are foreseen as the new trend in studies of biological sciences as the urgency to address biological dynamics are becoming more critical. The key aspects to successful live cell imaging are the appropriate optimization of microscopic settings, culture environment and fluorescent components. The most crucial issue is to provide an ideal imaging environment that not only sustained the normal cellular behavior but to prevent occurrence of cellular cytotoxicity. The latest development of new fluorescent dyes, proteins and biosensors give scientists a diverse range of options to track dynamics within the cell. Together with the emerging field of super-resolution microscopy, live cell bioimaging will enable observation of complex and intricate molecular interaction and interplay within the cell in greater details.

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8. References


