Imaging cellular signalling: many 'moving tales' in MAP kinase odyssey

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Cellular signalling events are at the core of every adaptive response. Signalling events link environmental changes to physiological responses, consequently allowing cellular and organismal sustenance and survival. Classical approaches to study cellular signalling have relied on a variety of cell disruptive techniques which yield limited kinetic information, while the underlying events are much more complex. In this article, we discuss how modern live cell imaging microscopy has found increasing utilization in revealing spatio temporal dynamics of various signalling pathways. Utilizing the well studied mitogen-activated protein kinase (MAPK) signalling cascade as a template, the design, construction and utilization of 'mobile' (translocation proficient) biosensors, suitable for studying MAPK signalling in living cells are described in detail. Experimental setup and results obtained from these biosensors, based on different proteins involved in the MAPK signalling cascade, have been described along with the setup of a microscope optimal for live cell imaging applications. Utilizing the ability to activate or deactivate signalling pathways using defined activators and specific pharmacological inhibitors, we also show how these sensors can yield unique spatial and temporal kinetic information of signalling in living cells.

Keywords: Cellular signalling, fluorescent protein, live cell imaging, translocation.

Introduction

'CELLULAR signalling' as a term describes a collection of events which leads to adaptive changes in a cell (consequently an organism) in response to perturbations in its external environment, allowing its survival and sustenance. This adaptation process, in general, is facilitated by three basic steps that involve (i) detection of the environmental cue, i.e. the stimulus, (ii) transmission of the stimulus from outside to inside the cell and (iii) generation of an intracellular response leading to adaptation. A simplistic sequence of these events can be listed as[1,2]

Detection → Transduction → Adaptation

These events collectively necessitate the presence of one or more components/molecules which can execute these distinct but linked events. In step 1, the detection process occurs through a 'receptor', generally a protein molecule present on the cell surface. The receptor molecule on interaction with a stimulus, typically a chemical entity, undergoes structural rearrangement, followed by its activation. The activated receptor then initiates step 2 of the pathway, wherein the signal is transferred from outside to the cellular interior through activation of transducer molecules, which can reversibly associate with the plasma membrane. The transducer molecules thus link the surface-anchored receptors to the effecter molecules, whose activation initiates step 3, consisting of initiation of adaptive responses through gene expression changes (Figure 1 *a*). Based on these distinct steps, it is not difficult to envisage that the 'transducers' should have the capability to move between two distinct cellular domains, plasma membrane and cytosol or other intracellular organelles (in case of eukaryotic cells). This dynamic relocalization termed as 'translocation' may not be revealed using standard snap-shot or end-point approaches; that is why these proteins are depicted as constrained to the specific subcellular locations. This problem is further confounded for transient and reversible events, which are often missed in standard assays. Over the years, it has been well established that using modern high-speed live cell imaging approaches, the unique spatial relocalization properties or 'translocation ability' of the transducer molecules can be effectively studied to reveal the spatio-temporal dynamics of various signalling cascades[3,4].

Translocation, a signalling necessity

Recently, a number of reports have shown that translocation of signalling proteins can lead to selective activation of downstream effectors in distinct cellular niches initiated as a result of physical relocalization of the transducer molecules[5]. Until recently, these relocalization or translocation events were studied using biochemical approaches like immunofixation and cell fractionation followed by Western blotting. As these experimental approaches require fixation or lysis of cells, only end-point analysis is possible and reversibility of translocation events yields heterogeneous information which is often difficult to interpret. Moreover, a recent study has
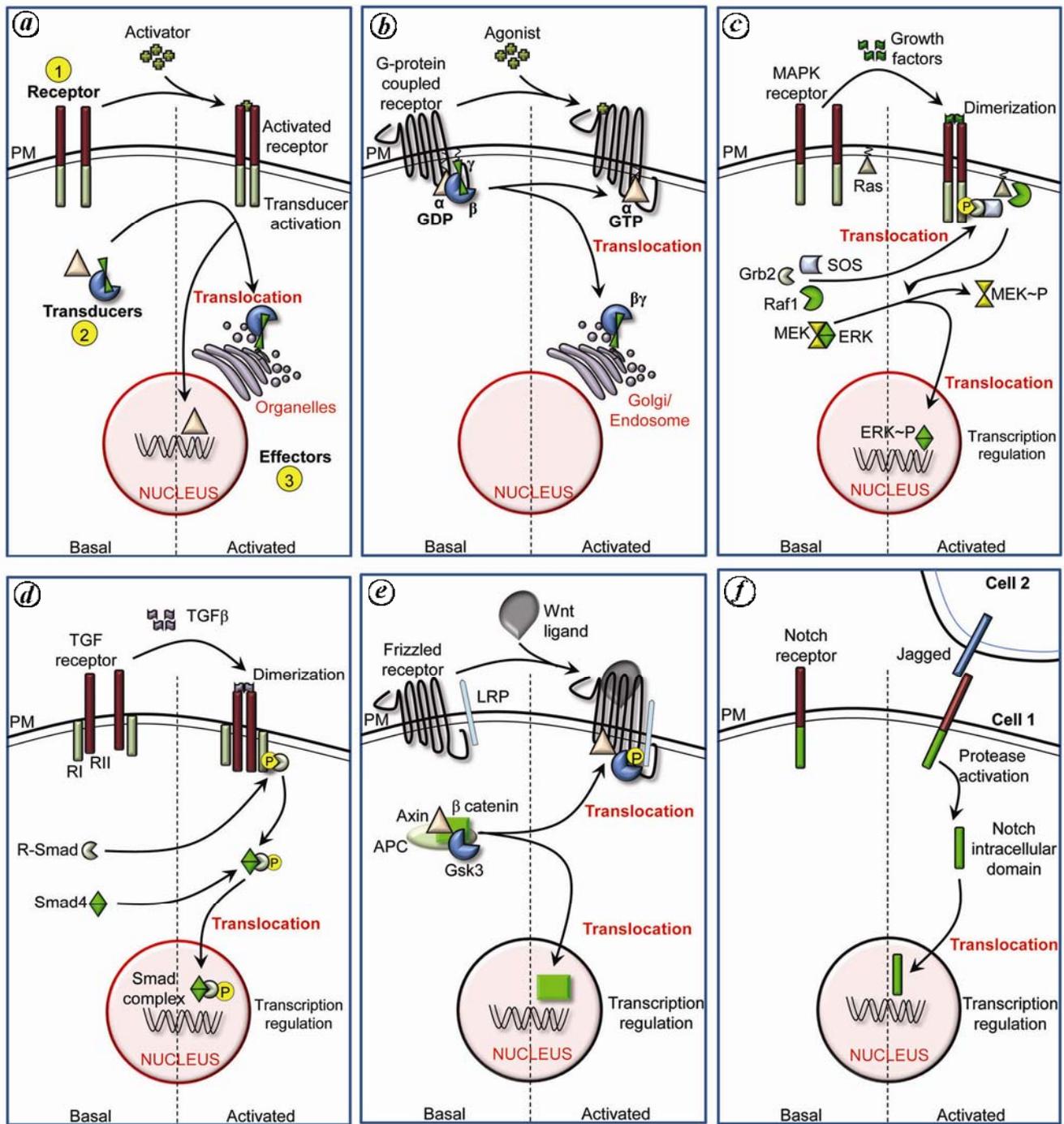
Figure 1. Schematics of different signalling cascades showing potential translocating proteins. The left section in each panel depicts the distribution of proteins in the basal state, whereas distribution in the activated state of the pathway is shown in the right section. ***a***, Organization of basic signalling events highlighting the three steps of detection, transduction and adaptation. ***b***, GPCR signalling cascade where G protein $\beta\gamma$-subunits are mobile transducers and translocate to endomembranes on receptor activation. ***c***, MAPK signalling cascade showing multiple transducer proteins such as Grb2, SOS, Raf1 and ERK1, whose locations are shown in basal as well as activated conditions. ***d***, TGFβ cascade showing translocation of Smad proteins in activated state; both transient and sustained translocation is represented. ***e***, Wnt signalling pathway showing spatial relocalization of Axin and β-catenin proteins on activation. ***f***, Notch signalling pathway showing the Notch transducer element, its generation and translocation post-activation.

also shown that fixation not only leads to mislocalization of native proteins, it also alters the cell morphology, highlighting the pitfalls associated with fixation studies[6].

In addition, these approaches mask spatio-temporal information and suppress cell–cell variations, which can be observed in individual cell studies[7,8].

Modern high speed techniques in microscopy have largely overcome these limitations by developing specialized live cell imaging methodologies, allowing studies into dynamics of signalling events[4,9–11]. Fluorescent protein tagged fusions of various signalling proteins have provided unique insights into the translocation behaviour of a large number of transducer proteins across different signalling cascades[9,12]. The following eukaryotic signalling cascades have one or more of such 'mobile' transducer molecules.

GPCR signalling cascade

These signalling pathways utilize guanosine-5′-triphosphate (GTP)/guanosine diphosphate (GDP)-binding transducer proteins which are activated by the membrane anchored 7-transmembrane (TM) or serpentine receptors called G-protein coupled receptors (GPCRs). Classically the 'mobile' components in these cascades are diffusible second messenger molecules such as cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP$_3$), Ca^{+2} etc. which in turn activate downstream effectors initiating cellular adaptation responses. Recently, translocation of G-protein $\beta\gamma$-subunits to intracellular membranes in a receptor activation dependent manner has been reported[13–15], providing another mobile component in the GPCR signalling cascade. Besides these proteins involved in the classical GPCR signalling, the non-canonical GPCR signalling cascade also has a few 'mobile' proteins such as β-arrestins and G-protein coupled receptor kinases (GRKs)[16,17] which are recruited to the GPCRs to deactivate the signalling. Figure 1 b shows the GPCR signalling pathway depicting translocation proficient proteins which can be used for studying the dynamics of GPCR signalling in live cells.

MAP kinase signalling

It is one of the most well studied signalling pathways and involves a large family of receptors, ligands, transducers and effectors proteins[18,19]. The ligands for these pathways are generally low molecular weight proteins and receptors having a single membrane spanning domain. Classically these pathways have been shown to regulate cell proliferation and hence are known as mitogen-activated protein kinase (MAPK) pathways. These pathways contain a large number of mobile protein components which relocalize on receptor activation. Figure 1 c depicts some of the components which show translocation[19–23].

TGFβ signalling

This is a simple but highly conserved signalling pathway mediated by a membrane anchored serine threonine kinase receptor. The pathway also contains many different mobile proteins which undergo both transient and sustained relocalization. Receptor activated Smad (R-Smad) proteins, which act as a transducer between transforming growth factor-β (TGFβ) receptor and transcription regulators, relocalize to receptor during activation and then translocate to the nucleus along with a cytosolic Co-Smad molecule. Smad4 is the common Co-Smad to all R-Smad and is essential for TGFβ signalling and can be used as marker for studying TGFβ signalling[24,25]. Figure 1 d describes this cascade, indicating Smad translocation events.

Frizzled/Wnt

This signalling pathway is critical for many development and differentiation processes. The receptor called 'Frizzled' is a 7-TM receptor, coupled to G-proteins as GPCRs, but utilizes different transducer molecules which include Axin, glycogen synthase kinase-3 (GSK3), casein kinase 1 (CK1) and β-catenin proteins. Unlike other signalling cascades where the balance of kinases and phosphatases regulates the outcome, spatial relocalization of the GSK3 kinase serves as both positive and negative regulator in Wnt signalling. In the basal state, GSK3 is present in cytosol bound to a complex of Axin, CK1, β-catenin and anaphase promoting complex (APC) proteins. Here, GSK3 phosphorylates β-catenin and targets it to degradation. On activation of Frizzled receptor, Axin, GSK3 and CK1 proteins are recruited to the receptor through a scaffolding protein, Disheveled, thereby preventing degradation of β-catenin, which in turn translocates to the nucleus to serve as a transcription factor[26]. During activation, GSK3 also phosphorylates the receptor and inactivates it to terminate the signalling. Figure 1 e depicts the pathways and translocation of Axin/GSK3/CK1 complex to the plasma membrane and that of β-catenin to the nucleus on receptor activation.

Notch signalling

In this pathway, both receptor and ligand are membrane anchored unlike other pathways where ligand is soluble. This arrangement ensures that the signalling only takes place when receptor expressing cells and ligand expressing cells are juxtaposed to each other. The activation step induces proteolytic cleavage of the receptor, leading to dissociation of Notch intracellular domain (NID) in the receptor cell, which translocates to the nucleus to serve as a transcription factor[27,28]. Figure 1 f depicts the translocation of NID to the nucleus.

Translocation and limitations of existing techniques

It is evident from the pathways described above that translocation events are necessary for proper execution of

a signalling event. But identifying and studying these events using conventional biochemical techniques have always been a challenge, mainly due to the following reasons:

- immunofixation approaches alter the native localization of proteins, during the steps of cell permeabilization or fixation,
- it is difficult to track multiple events at the same time in the same population of cells,
- immunoblotting studies based on ensemble methods mask the cell–cell variations and provide information only from a larger subset of cells,
- cell-sorting methods like fluorescence activated cell sorter (FACS) allows individual cell analysis, but are limited in temporal resolution,
- for many signalling proteins, high affinity antibodies are difficult to generate,
- different fixation protocols have to be followed for different antibodies and hence the execution tends to be cumbersome.

In light of these limitations, there have always been demands for techniques which can overcome them and can also yield dynamic spatio-temporal information about the cascades, especially during the course of their activation and deactivation.

Imaging cellular signalling: overcoming the spatio-temporal limitations

Over the last few years, microscopy techniques have developed significantly to overcome the spatial limitations of imaging the biomolecules inside a cell and the temporal limitations have also been answered more recently[9,29–34]. For temporal studies of a signalling event, the best methodology should provide the ability to study the event as it is happening, i.e. 'live viewing'. This has become possible by genetic fusion of the protein of interest with fluorescent proteins, allowing the visualization of their behaviour in living cells using fluorescence microscopy. These 'imagable' fusion proteins have been termed as 'biosensors' as they sense and report the biological activity of a protein. Using this design, a large number of translocation-proficient biosensors have been developed for various signalling cascades, allowing studies into both spatial and temporal dynamics of signalling events in live cells[3,9,11]. Live imaging approaches tend to be better because

- they allow analysis of transient events,
- quantitative kinetics of pathway activation based on kinetics of protein relocalization can be obtained,
- spatial restriction of protein activity can be obtained, even if transient,
- multiple events can be studied in the same cell (using a combination of fusion proteins),

- cellular heterogeneity can be identified and quantitated,
- cellular events can be studied in the native environment of a cell in the backdrop of multiple interaction networks,
- experiments can be done with a very small number of cells, critical where biological material or execution limitations exist. For example, imaging can be done with transiently transfected cells and with primary cells,
- spatio-temporal dynamics of various proteins can be studied during the course of pathway activation and deactivation.

Overall, more significant observations can be made with much less resources and time using live cell imaging approaches compared to any other classical techniques. Based on these advantages it is expected that microscopy will be beneficial for studying signalling events. However, due to extensive choices available now, it is usually difficult to choose the right method. Confocal microscopy, using which the optical sections of samples can be obtained, provides better spatial resolution in the z-axis and provides better signal-to-noise ratios. However, owing to the nature of point-by-point laser scanning employed in confocal microscopy, it tends to be slow and requires strong laser beam to image. These parameters make confocal microscopy unsuitable for live imaging applications, as it increases the time taken to image a sample and induces phototoxicity which interferes with signalling studies. Overall, a widefield microscopy setup with high-efficiency detector is preferred as it does not have these limitations. The image resolution obtained using this setup is however limited; but now-a-days it can be improved by utilizing deconvolution algorithms which significantly reduce background noise by subtracting out-of-plane light. In the subsequent sections we will describe the design and setup of a widefield, live cell-imaging microscope suitable for imaging cellular signalling events.

Microscopy and live cell imaging

The first description of microscopy in biology dates back to an influential compendium published by Robert Hooke in his book Micrographia. In his testament, he presented the morphological features of a large number of biological entities including the eye of a fly, a louse and a plant cell amongst many others using a simple magnifying glass. But perhaps, it was Leeuwenhoek, who first observed 'living' samples under a microscope, in the form of a moving amoeba and paramecium. Much later, Robert Brown described the first organelle, i.e. nucleus and Gerlach introduced the concept of staining of the biological specimens leading to the development of differential stains for demarcating different subcellular structures.

Though, the discovery of electron microscopy allowed us to study the intracellular structures with unprecedented details, it was the usage of fluorescent stains and fluorescence microscopy which enabled visualization of the microscopic world hidden inside a cell within the reach of every laboratory. Subsequently, the development and utilization of green fluorescent protein (GFP)[35] and its spectral variants as a fusion partner have modernized the conventional fluorescence microscopy by enabling studies on protein behaviour inside living cells, which includes studying their movement, trafficking, localization alterations, intramolecular interactions and *in vivo* stability amongst many others processes[36–38]. It is now well accepted that most biomolecules are not static and the dynamics of their intracellular movement regulates various aspects of cellular physiology[4,11,12]. Here we present the MAP kinase signalling cascade as a case study, to describe the design and biological considerations for imaging signalling events in a living cell.

Translocation case study: MAPK cascade – a translocation minefield

One of the best characterized signalling cascades is the MAPK which deals with growth factor signalling and regulation of cellular proliferation. There are three broad MAPK cascades, distinguished by receptors and the effector proteins activated in them, viz. extracellular signal-regulated kinase (ERK)1/2 cascade, p38 MAPK cascade and Jun N-terminal kinase (JNK) cascade. The ERK1/2 pathway, also called the classical 'MAPK' cascade, contains many mobile transducers like Grb2, Raf1, ERK1/2, etc.[18,20,39–41]. These proteins relocalize on receptor activation to different intracellular locales where they initiate subsequent steps in the pathway. Using the MAPK pathway as a model system, the following sections describe how the dynamics of cellular signalling can be studied using live cell microscopy techniques. The protein components which are biologically well characterized for their translocation behaviour in this pathway include

- *Grb2 and son of sevenless (SOS) proteins*: Activation of growth factor receptors leads to their autophosphorylation onto which Grb2 and SOS proteins can bind. Hence in the basal condition, these proteins are present in the cytosol and but on activation relocalize to the plasma membrane[42]. Translocation biosensors developed using SH2 domains present in Grb2 proteins show spatial relocalization from cytosol to plasma membrane on receptor activation.

- *Raf1:* This protein interacts with Ras proteins, which are activated by Grb2/SOS proteins (mentioned above). It resides in the cytoplasm under basal condition and on pathway activation translocates onto the plasma

membrane for Ras–Raf interaction[43]. Translocation behaviour is similar to Grb2/SOS proteins.

- *ERK1/2:* It is the terminal transducer protein in the MAPK cascade, which is activated by cytosolic MAPK/ERK kinase (MEK) protein and in basal state is retained in the cytosol by inactive MEK. Through the Ras–Raf–MEK cascade, ERK is activated by a phosphorylation event which dissociates it from MEK, allowing its relocalization to the nucleus. It is one of the most popular sensors for detecting MAPK activation in living cells and shows cytosolic distribution in the unstimulated cells and localizes to the nucleus under active condition[20,22].

- *Ets2 repressor factor (ERF):* ERF is a nuclear protein which on phosphorylation by nuclear ERK1/2 exits the nucleus, allowing transcription initiation[44]. The biosensor developed using ERF demonstrated relocalization profile opposite to that of ERK; while ERK translocates from cytosol to nucleus, ERF translocates to cytosol from nucleus.

Using these protein biosensors, we describe the microscopy set-up to image them and how these sensors generate more quantitative information for significantly less effort using live imaging set-up.

Live cell imaging: a step-by-step guide

The most important parameter in live cell imaging studies involves keeping the cell alive without introducing stress during the course of the experiment. The issue is more important while studying signalling events because stress-mediated signalling can alter observations of many other signalling pathways. Hence, the following steps have to be considered for developing and using translocation biosensors to study signalling events.

Step 1 – Design of translocation biosensors: Using the epidermal growth factor receptor (EGFR) signalling as an example of a typical MAPK signalling cascade (Figure 2), one can see that there are a number of molecules which are potential candidates for generating sensors, based on changes in their spatial distribution over time after receptor activation. By genetic fusion of the full length or bioactive domains of these proteins with a fluorescent protein tag, a 'biosensor' can be generated, whose spatio-temporal dynamics can be imaged in living cells using fluorescence microscopy.

For developing such biosensors, aspects which need to be considered include choice of fluorescent protein (FP) and location of its fusion with the protein of interest. A large spectrum of fluorescent proteins is now available, making selection quite complicated[37,45–48]. The choice of fluorescent protein selection depends on the number of
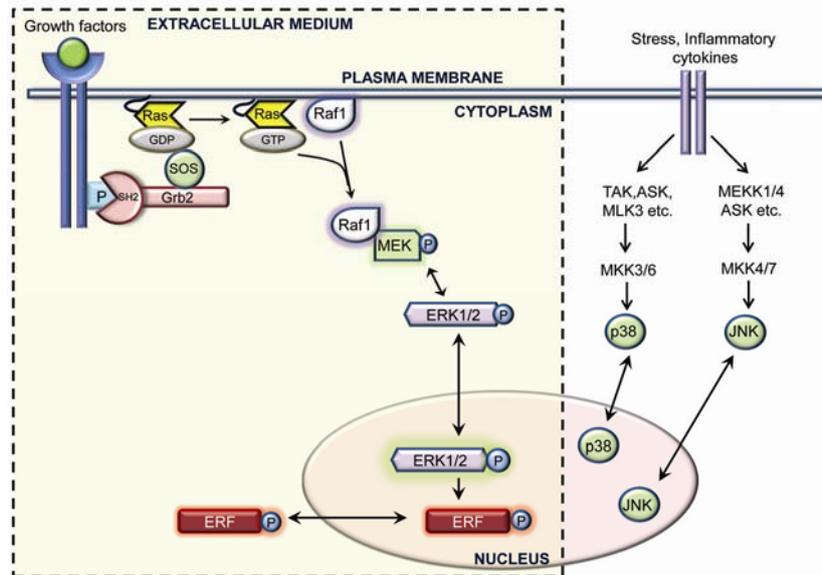
Figure 2. MAP kinase signalling cascades. A graphical representation of three main MAP kinase pathways, showing proteins capable of translocation and their spatial localization under basal and activated states. The left box depicts the MAPK–ERK pathway in detail, showing many mobile proteins which translocate on activation.

proteins whose behaviour will be studied in the same cell. Generally for imaging a single protein, GFP is used as a fusion protein of choice on account of its well characterized features, which include high photostability, fast maturation kinetics, high quantum yield and less toxicity[49]. For multispectral imaging, fluorescent proteins with different excitation and emission wavelengths such as BFP (blue), CFP (cyan), YFP (yellow), RFP (red), mCherry (cherry red) and mOrange (orange) amongst many others can be used[47,48]. Typically fluorescent proteins, spectrally well separated in terms of their excitation and emission maxima, are used for simultaneous imaging of multiple proteins[48], e.g. GFP and RFP for imaging two proteins and BFP, YFP and mCherry for three proteins.

Comparatively, the choice of location of the fusion between the protein under analysis and FP tends to be more difficult to decide. Using structural information or cellular localization information of the proteins, the FP tag is fused on a location where it does not interfere with the functional activity of the target protein[50,51]. Generally, the proteins are tagged either at C or at N-terminus of the FP and the fusion which retains the functional activity and does not show mislocalization in cells, is used for subsequent studies[51].

Step 2 – Live cell microscopy set-up: As described earlier, for obtaining spatio-temporal dynamics of signalling proteins within the rapid time frame of signalling events, the live cell microscopy setup should be designed to minimize stress to ensure that no other signalling pathway is activated. During imaging, stress can be generated by suboptimal sample environment, phototoxicity and thermal fluctuations. The problem is further confounded by inherent issues with autofluorescence and low signal-to-noise ratio in living samples that demands longer exposure duration for living samples.

For imaging cell signalling events, an inverted fluorescence microscope is recommended as it allows imaging of cells directly on the cell culture dishes (glass bottom) or in the imaging chambers containing cells immobilized on glass cover slips. In addition to the basic microscope, other components are added to overcome phototoxicity, loss of focus, environmental fluctuations, thermal/temperature drift of the sample which interfere with long-term imaging of living samples. The setup of a typical epifluorescence microscope for live imaging is shown in Figure 3 alongwith that of a live imaging chamber. We describe below the imaging parameters and hardware components which are used to obtain optimal imaging readouts in a live cell imaging experiment:

(i) Signal-to-noise ratio: It is known that exposure of cells to intense light leads to phototoxicity, especially when dealing with fluorescent proteins which generate reactive oxygen species, affecting signalling pathways[34]. To overcome this, imaging is done with minimal exposure of cells to light. This is achieved using a combination of intense light sources such as lasers for very short durations for sample excitation, with high quantum efficiency electron multiplying charge coupled device (EMCCD) cameras, which have greater than 90% photon collection capability. Along with this, high numerical aperture (NA) lenses are also used to ensure that most of photons emitted by the sample are collected and directed
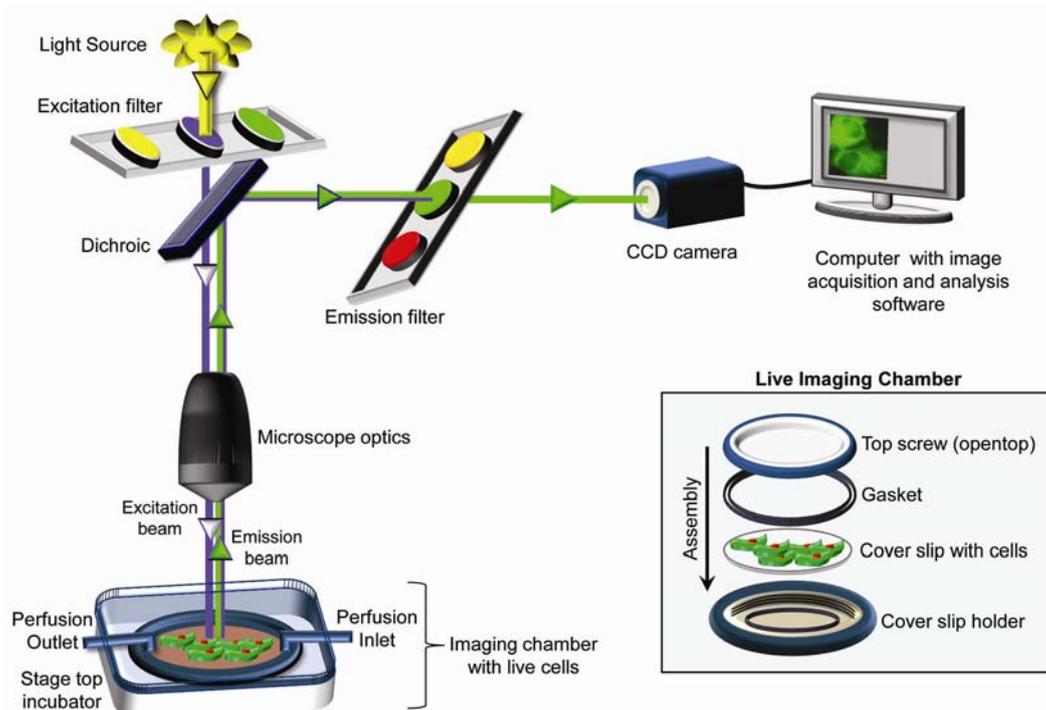
Figure 3. Microscope and live imaging chamber set-up for studying dynamics of signalling events in live cells. Graphical representation of a fluorescence microscope set-up equipped with devices to make it suitable for live imaging applications as described in the text. For imaging individual fluorescent molecules, first appropriate excitation wavelength from the white light is selected using a high-speed bandpass filter. The light is guided through a dichroic beamsplitter, which allows transmission of excitation light only and serves as a mirror for the emitted fluorescent light. The excitation light is guided through the microscope optics through the objective to the sample. The sample (cells) is kept in an imaging chamber linked to a perfusion system housed in a stage top incubator for maintaining appropriate physiological conditions. On excitation of the sample, the fluorescence emission along with the reflected excitation light is collected by the microscope optical train. The light is then reflected using a dichroic mirror and sent to an emission filter, allowing only emission light to reach the CCD camera. The computer is used to control all the attached devices, set up the image acquisition protocol and analyse the acquired data. The box on the right shows the assembly of an imaging chamber for imaging cells using a disposable glass cover slip.

to the camera. More recently, light emitting diode (LED) light sources are finding increasing use in fluorescent microscopy as they have very long life and do not generate heat, which can cause cellular stress.

(ii) Loss of focus: In live imaging applications, the loss of focus is a frequent constraint for long-term observations. This happens due to a variety of reasons which include changes in thermal currents, cover-slip flex, gravitational strain on the *z*-drive, electronic fluctuations and liquid/media evaporation. To overcome this, a number of microscopes now contain an inbuilt drift compensation system, which actively compensates plane shift by driving the *z*-axis motor to a fixed optical plane, continuously monitored using an IR laser. This system allows long term imaging of the sample without loss of focus and is an essential component for live imaging set-ups.

(iii) Cell health and viability: Keeping cells alive and healthy during imaging experiment is highly imperative as stress activated pathways interfere with almost all signalling events. On a microscope, the cells under analysis can be kept healthy by placing them inside a CO_2 incubator which can either be on microscope stage (stage incubator) or can enclose the entire microscope (cage incubator). Both the setups allow incubation of cells at optimal temperature (maintained by heaters); CO_2 concentration and humidity. To suit all these requirements the design of imaging chamber should introduce minimal environmental perturbations, should offer excellent optical properties for quick focusing and minimize photobleaching of the sample. Currently, the imaging chambers range from simple glass cover slip chambers, glass bottom dishes to sophisticated perfusion system, all of which can be housed inside an environmental incubation system.

(iv) Cell stimulation: As signalling events are studied both before and after receptor activation using pharmacological entities such as agonists and antagonists, a controllable perfusion and fluid delivery system is a vital addition to the imaging setup. A typical perfusion system consists of syringes linked to a controllable valve system
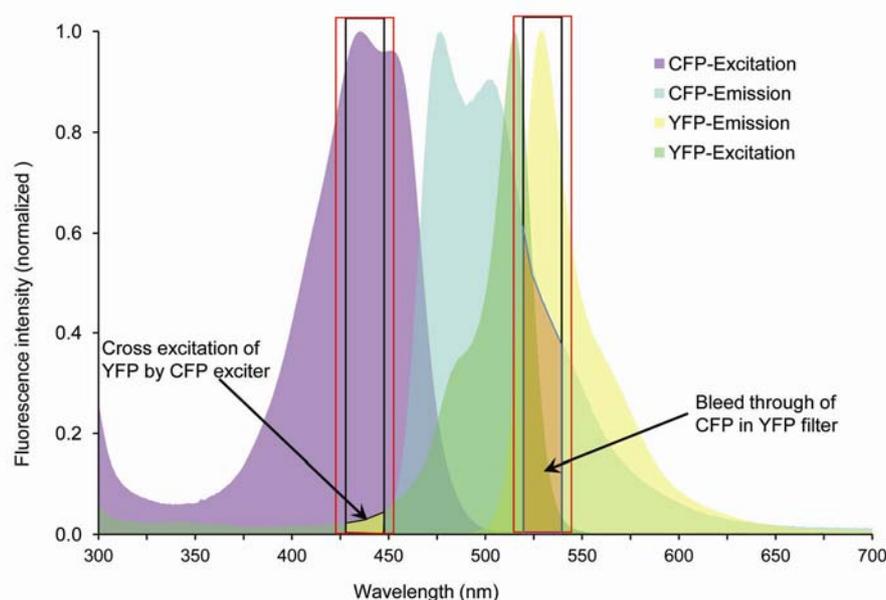
Figure 4. Cross-excitation and bleed-through effects during imaging fluorescent proteins. Excitation and emission spectra of CFP and YFP fluorescent proteins are shown. The marked area under the curves represents cross-excitation of YFP from CFP excitation light and bleed-through of CFP emission in YFP emission filter. The boxes (black and red) represent the bandwidth of the filters. Note that the effect of spectral overlaps increases as the band width of filters is increased (narrow band width filter, black box and wider band width filter, red box). The spectra of CFP and YFP fluorescent proteins were obtained from the fluorescent protein database hosted at http://www.tsienlab.ucsd.edu/Documents.htm and are used here for representation purpose only.

housing the pharmacological agents. These syringes are connected to a flow rate controller and delivery manifold to directly deliver the stimulations to the cells in the imaging system or dish. Using a closed perfusion system, the entire milieu of the cells can be exchanged within a few seconds of solution switching, allowing rapid activation and deactivation of signalling events.

(v) Multi colour imaging: For imaging of proteins tagged to different fluorophores, a device allowing rapid switching between both excitation and emission wavelengths without introducing any vibrations in the imaging set-up is essential. In a standard microscope this is achieved using a motorized filter turret which switches between selected filters. These turrets tend to be slow and can be the limiting factor in high speed multi colour imaging. To overcome this, external high-speed filter wheels are used which are capable of switching between selected wavelengths in as little as 50 ms. This rapid switching allows imaging of dynamics of many molecules without compromising on temporal information. While using multiple fluorophores in a single experiment, it is important to select appropriate band filters to ensure minimum cross-excitation and bleed-through artifacts observed without compromising on signal-to-noise ratio. Figure 4 shows how cross-excitation and bleed-through can affect the readout obtained from fluorescent proteins.

(vi) Experimental control and data analysis: Last, but not the least, a sophisticated and customizable software is needed to control all the devices and units mentioned above to perform high-speed 3D (*XYZ*), 4D (*XYZT*), 5D (*XYZT* multi-colour) and 6D (*XYZT* multi-colour multi-site) imaging. A number of commercial software options are available to perform this and most microscope vendors sell their own software. Importantly, sophisticated algorithms should be available in the software to calculate information like colocalization index, intensity change over time, etc. alongwith a deconvolution software to enhance signal-to-noise ratio.

Step 3 – Imaging signalling events: Using the microscope set-up for live cell imaging experiments, the signalling events can be studied using biosensor constructs as imaging tools. During the experiment the cells are kept healthy and stress free by imaging them under a well-defined medium with appropriate CO_2 and temperature conditions, so that any stimulus potentially capable of triggering signalling event is not present. More commonly, for MAPK signalling the cells are grown in media devoid of serum for a few hours before imaging to bring the cells to a resting state. These rested cells are then subjected to stimulations which activate or deactivate MAPK signalling pathways.

The results of imaging some of the events in MAPK pathway in resting cells which have been stimulated with
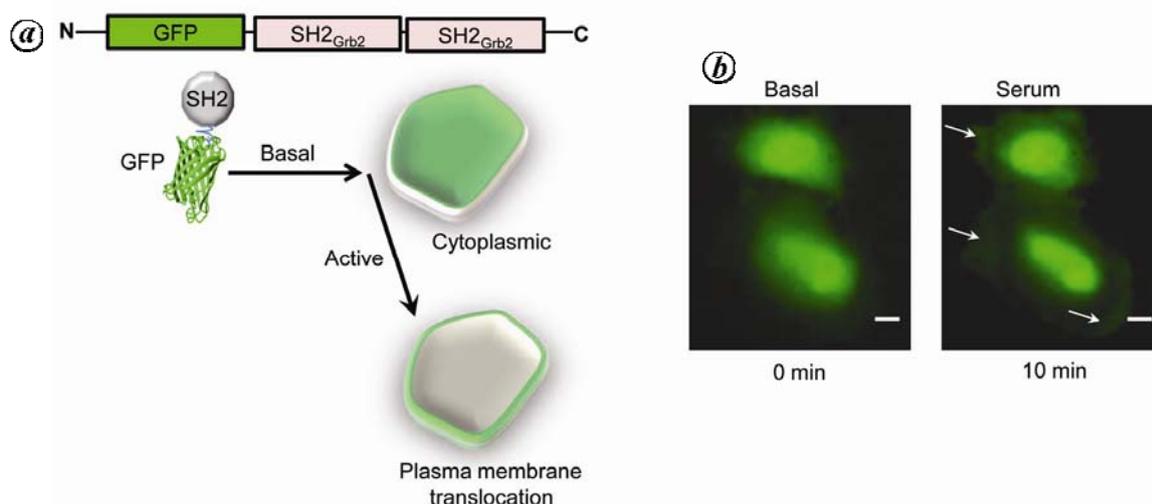
Figure 5. Design and translocation behaviour of SH2 biosensor. **a**, Design of SH2 biosensor. Graphical representation of the fusion protein, generated by fusing tandem SH2 domains from Grb2 protein to the C-terminal of GFP protein and its scheme of translocation from cytosol to plasma membrane on activation. **b**, Translocation of SH2 biosensor in living cells. HeLa cells were transiently transfected with GFP-tagged SH2 biosensor and serum starved for 3 h before imaging. The cells were stimulated with serum and imaged for 10 min at 37°C and 5% CO_2 to activate MAPK signalling. Images showing localization of GFP–SH2 in a live cell before stimulation (0 min) and after serum stimulation at 10 min. Images are shown for one representative cell (scale 10 μm, magnification 40×). Arrows depict the region on plasma membrane where intensity changes are maximal.

serum to initiate signalling have been described below. It is assumed here that since MAPK signalling occurs in almost all cell types; these events can be studied in various cell types independent of their lineage.

(i) Imaging Src homology domain2 (SH2) biosensor to monitor the EGFR activation: As described above, on activation of receptor tyrosine kinases (RTKs) such as EGFR, the Grb2 protein docks to the plasma membrane through interaction of its SH2 domain with phosphorylated receptor, leading to its relocalization from cytosol to plasma membrane. By fusing the SH2 domain of Grb2 with a FP, a sensor can be generated, which is recruited to the plasma membrane on receptor activation as shown in Figure 5 a. This translocation of SH2 biosensor is an efficient indicator of an activated RTK pathway as shown in Figure 5 b, where it is imaged in response to serum stimulation in HeLa cells. The SH2 biosensor used has been engineered by fusing two tandem SH2 domains (for enhanced signal amplification) at the C-terminal domain of GFP protein (Figure 5 a).

(ii) Imaging ERK1/2 translocation biosensor as an indicator for MAPK pathway activation: Activation of MAPK cascade culminates in the activation of ERK1/2 proteins which relocalize to the nucleus on phosphorylation-mediated activation. Biosensor based on the translocation ability of ERK1/2 protein, serves as a potent indicator of the presence of functional MAPK cascade. The sensor is constructed by fusing ERK1/2 proteins with the FP at its N-terminal domain (Figure 6 a). The generated sensor

demonstrates cytosol to nuclear translocation in Chinese hamster ovary (CHO) cells on serum stimulation, as shown in (Figure 6 b and c).

(iii) Imaging ERF translocation biosensor as a marker for MAPK transcriptional changes: ERF protein, also called as Ets2 repressor factor, acts as a transcription suppressor. On stimulation it translocates out of the nucleus on phosphorylation by an active ERK1/2, to bring about changes in gene expression. ERF has been used as a biosensor by fusing it to the C-terminal domain of mCherry fluorescent protein (Figure 7 a). Imaging dynamics of this protein in COS 7 cells in response to serum stimulation showed translocation from nucleus into the cytoplasm (Figure 7 b), hence serving as an indicator of transcriptional activation associated with MAP kinase activation.

In addition to time-lapse imaging to identify and study translocation events, microscopic techniques to study transient changes in protein–protein interactions during the course of a signalling cascade have also been used to study cell signalling events. Techniques such as Förster resonance energy transfer (FRET) and fluorescence life-time imaging microscopy (FLIM) have been extensively used to record the changes in fluorescence intensity and lifetime of two interacting fluorophores, which are affected by the degree of interaction between the proteins to which they are tagged. Similarly, to demonstrate the dynamics of protein movement in living cells, techniques like fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are also
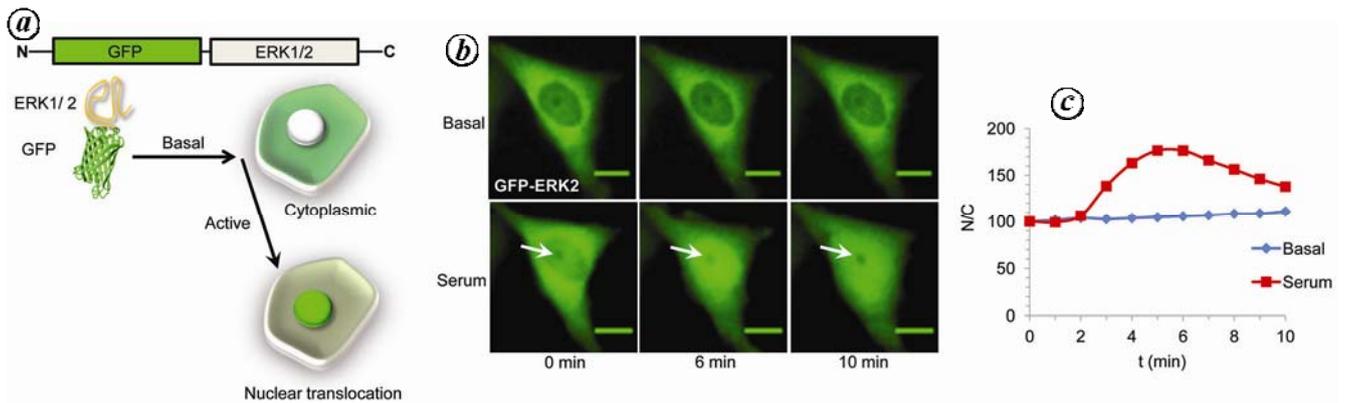
Figure 6. Design and translocation property of ERK1/2 biosensor. *a*, Design of ERK1/2 biosensor. Graphical representation of the fusion protein, generated by fusing GFP to the N-terminal of the ERK1/2 protein and the scheme of its translocation from cytoplasm into nucleus upon activation. *b*, Nuclear translocation of ERK2 biosensor. CHO cells transiently transfected with GFP-tagged ERK2 and untagged MEK protein were serum-starved for 1 h and then stimulated with serum and imaged for 10 min. Images show localization of EGFP–ERK2 in a live cell before stimulation (0 min) and after serum stimulation at 6 and 10 min time intervals. *c*, Plot showing the change in nuclear by cytoplasmic fluorescence intensity of a fixed region (N/C intensity) over time. Images and plot are shown for one representative cell (scale 20 μm, magnification 40×).
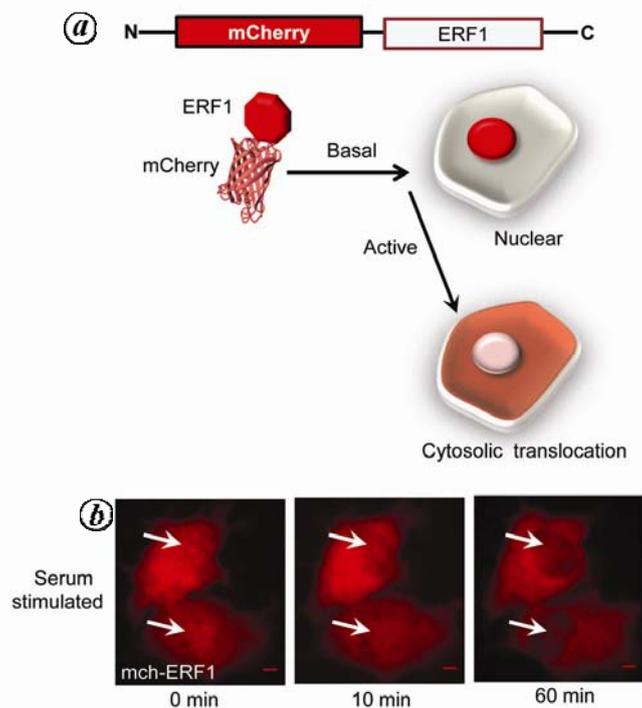
Figure 7. Design and translocation property of ERF biosensor. *a*, Design of ERF biosensor. Graphical representation of the fusion protein generated by fusing ERF protein with mCherry FP at its N-terminal end and the scheme of its spatial relocalization from cytoplasm to nucleus on activation. *b*, Nuclear translocation of ERF transducer. COS7 cells transiently transfected with mCherry-tagged ERF were serum-starved for 3 h before imaging and then stimulated with serum and imaged for 60 min at 37°C and 5% CO_2. Images show localization of mCherry–ERF in a live cell before stimulation (0 min) and after serum stimulation at 10 and 60 min. Images are shown for one representative cell (scale 10 μm, magnification 40×). Arrows depict the region in the nucleus where intensity changes are observed.

used. These techniques are useful when the distribution of proteins does not show much change overtime, but it is the dynamics of their distribution which shows alteration. Similar to the translocation methods described above, these techniques also require generation of a fluorescently tagged variants of proteins under analysis.

FRET and FRAP in studying MAPK signalling

FRET is based on the principle of transfer of non-radiative resonance energy from one chromophore called the 'donor' to another chromophore, called the 'acceptor'. The efficiency of this energy transfer, known as

FRET efficiency, is primarily dependent on the distance between two chromophores and their spectral overlap. FRET is observed when two chromophores come in the vicinity of each other during protein–protein interaction resulting in either loss (for donor) or gain (for acceptor) of fluorescence. Examples of such FRET biosensors include cameleon sensor[52] and its variants[53] for studying calcium signalling, Raichu–Ras sensor for studying Ras signalling[54] and an extracellular signal-regulated kinase activity reporter (EKAR) sensor[55] for studying ERK signalling in real time in living cells, amongst many others. In EKAR sensor, EGFP molecule, used as a donor species is fused with monomeric red fluorescent protein1 (mRFP1) molecule, which serves as an acceptor. Between them a long, 72 amino acid linker along with ERK substrate-specific phosphorylation sequence is introduced. On phosphorylation of this domain by ERK, the two fluorophores come close to each other and show FRET, i.e. show transfer of energy from EGFP/CFP to mRFP1/YFP, which does not happen when no ERK activity is present. Using this technique, ERK activity can be monitored in living cells robustly and reversibly.

Though FRET is a popular technique to study the kinetics of a protein–protein interaction, it has its own limitations in studying molecular diffusion within different cellular compartments in a cell. It has been shown that diffusion of various proteins might be altered on activation of various signalling pathways. In order to study such processes, FRAP is used for measuring diffusion kinetics within cytosol as well as between different subcellular compartments inside a cell. FRAP involves photobleaching a fluorophore in a small region of interest (ROI) within a cell, followed by monitoring the recovery of fluorescence within this ROI, which serves as an indicator of diffusion of the tagged molecule from other unbleached areas. The technique has been used successfully to measure the mobility of Ras and Rap1 in neurite cells in response to EGF stimulation[54] and to measure ERK shuttling in the MAP kinase signalling under unstimulated or stimulated conditions[56].

These examples demonstrate that biosensors based on various participating proteins in a signalling cascade can serve as potent tools for studying as well as quantifying various signalling cascades. As evident by the experiments described above, the sensors can consistently reveal temporal dynamics of the signalling cascade without the need of experiments performed at different time points. Further, the spatial dynamics of the proteins revealed by imaging also provides information about events, which is otherwise lost in the conventional analysis.

Concluding remarks

Cellular signalling has always been a field of curiosity in biology, warranting better insight into the 'sensing and responding' mechanisms underlying various physiological conditions. Inside each cell, a signalling event typically involves spatial translocation of one or more molecules, shown to be critical in eliciting a specific physiological response. With the development of modern live cell imaging and sensitive fluorescence microscopy techniques, these events can now be studied in real time in a cell and more reliable information compared to the conventional approaches can be obtained. It is anticipated that in future new imaging techniques such as super-resolution microscopy and single molecule imaging will shed more light on the intricacies of signalling events in living cells.

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