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REVIEW

Visualization of the spatial and temporal dynamics of MAPK signaling using fluorescence imaging techniques

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Abstract Conserved mitogen-activated protein kinase (MAPK) signaling pathways are major mechanisms through which cells perceive and respond properly to their surrounding environment. Such homeostatic responses maintain the life of the organism. Since errors in MAPK signaling pathways can lead to cancers and to defects in immune responses, in the nervous system and metabolism, these pathways have been extensively studied as potential therapeutic targets. Although much has been studied about the roles of MAPKs in various cellular functions, less is known regarding regulation of MAPK in living organisms. This review will focus on the latest understanding of the dynamic regulation of MAPK signaling in intact cells that was revealed by using novel fluorescence imaging techniques and advanced systems-analytical methods. These techniques allowed quantitative analyses of signal transduction in situ with high spatio-temporal resolution and have revealed the nature of the molecular dynamics that determine cellular responses and fates.

Keywords MAPK · Fluorescence imaging · Systems analysis · Environmental response

Introduction

Intracellular signaling events in cells of living organisms regulate a vast array of cellular functions to maintain the life of the organisms. Cells must obtain proper information about the microenvironment surrounding them and adapt to this by inducing gene expression, cellular proliferation, differentiation or even cell death. Cells sense chemical or physical environments, pass this information to the inside of cells through the plasma membrane, process information according to the cellular context and induce a proper cellular response during the decision-making process.

Accumulating evidence based on genetic and molecular biological studies has proven that a large number of molecules are involved in this process. More recent progress in high-throughput analysis of cellular signaling molecules has added evidence of complex connections and regulations among molecules that comprise a huge intracellular signaling network whose regulation is far beyond our imagination. Due to the technical limitations of the previous methods used for analysis, how those molecules behave in cells of living intact animals has long been unknown.

In the last 3 decades, application of fluorescent proteins in analyzing intracellular signaling by fluorescence imaging has been steadily developed and improved. As a consequence, the spatiotemporal dynamic behaviors of these signaling molecules are finally beginning to be unraveled. The aim of this review is to survey recent advancements in our understanding of the regulation and function of the dynamically behaving signaling molecules that occur in vivo. Based on recent studies on MAPK signaling dynamics, how and why cells utilize such dynamic signaling will be described. The imaging methods indispensable for analyzing the dynamic signaling in vivo will be also introduced in this review.

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Versatile MAPK signaling in eukaryotes

A conserved three-tiered cascade of kinases

Signaling by the MAPK family is a major mechanism through which cells respond to a variety of stimuli from the extracellular environment [1, 2]. Molecules that comprise the core of MAPK signaling are evolutionarily conserved among eukaryotes from yeasts to mammals. MAPKs are activated via a three-tiered cascade of kinases, which is composed of a MAPK, a MAPK kinase (MAP2K) and a MAPK kinase-kinase (MAP3K). Several subfamilies of MAPK cascades coexist in mammalian cells: the growth-promoting extracellular signal-regulated kinase (ERK) family and growth-suppressing stress-activated protein kinase (SAPK) families, namely the c-jun N-terminal kinase (JNK) and p38 families. The relatively recently discovered ERK5 is also ubiquitously expressed in mammalian cells. In addition, atypical MAPK families (ERK3/4, ERK7/8) that have distinct regulation and functions were also discovered. Although we mainly focus on conventional MAPKs in this review, interested readers are encouraged to consult comprehensive reviews [3, 4].

Regulation of MAPK subfamilies

Of these MAPK family cascades, the ERK family cascade is the best studied. In the ERK cascade, a cell surface receptor is first stimulated by a growth factor (i.e., a mitogen). The activated receptor (typically a tyrosine kinase-type receptor such as epidermal growth factor receptor, fibroblast growth factor receptor or platelet-derived growth factor receptor) then induces activation of the small G protein Ras, which activates the MAP3K Raf. Activated Raf then phosphorylates its cognate MAP2Ks (MEK1/2), which subsequently phosphorylate a downstream MAPKs (ERK1/2). In this manner, information from growth factors or mitogens outside the cells is transmitted to the cytoplasm and the nucleus in the form of activated ERK.

The JNK and p38 MAPK family cascades are initiated by physiological mediators such as transforming growth factor-ß (TGF-ß), tumor necrosis factor-α (TNF-α) and interleukin-1ß (IL-1ß) as well as by environmental (physical and chemical) stresses such as ultraviolet light, gamma rays, translation inhibitors, hyperosmotic stress and oxidative stress. Similarly to the ERK cascade, small GTPases act upstream of the p38 and JNK cascades, but in these cases the relevant GTPases are Cdc42, Rac and Rho. The specific stress-activated MAP2Ks are activated by diverse MAP3Ks including MEKK1/2/3, MTK1, TAK1, ASKs, MLKs and TAOs [5]. The p38 kinase is activated mainly by the MKK3 and MKK6 MAP2Ks, whereas JNK is activated by the MKK4 and MKK7 MAP2Ks. Involvement of a large number of upstream stress MAP3Ks in these cascades presumably reflects the diversity of the physiological mediators and stress stimuli that activate these cascades [6]. However, the molecular mechanisms by which various stress stimuli activate MAP3K are still largely unknown. Many of the stress MAP3Ks are shared in the JNK and p38 pathways, and some of them (MEKK2/3) are also commonly utilized in the ERK5 pathway. Actually, ERK5 can respond to several types of stresses as well as to growth factors [4] (Fig. 1).

Specific docking domains and scaffold proteins

The specificity of a signaling cascade should be maintained throughout the cascade. It is therefore of interest to determine how the signal of an activated MAP3K is correctly transmitted to its cognate MAP2K and MAPK and how closely related (homologous) MAP2Ks and MAPKs are discriminated against. In general, the interaction between the catalytic center of a kinase and phospho-acceptor site of a substrate is an important factor for determining the specificity of their interaction. Indeed, mammalian MAPKs specifically phosphorylate a Ser/Thr-Pro motif in their substrates, and MAP2Ks phosphorylate threonine and tyrosine residues in the Thr-Glu-Tyr, Thr-Gly-Tyr or Thr-Pro-Tyr motifs of their substrates [7, 8]. In addition to the substrate site specificity, several other mechanisms ensure a specific kinase-substrate interaction in the MAPK cascades. A docking domain located at the C-terminus of MAP2Ks (the DVD domain) allows specific interaction between MAP3Ks and their cognate MAP2Ks [9]. Similarly, a conserved CD docking domain of MAPKs binds to the MAPK-binding domain (the D-domain) of the MAP2Ks, MAPK phosphatases and MAPK substrates [10]. In addition to the D-domain, ERK-MAPK recognizes another docking motif (DEF domain), and both the D-domain and DEF domain contribute to binding specificity to ERK [4]. In fact, the fidelity of the signaling cascade can be achieved by specific docking interactions between the kinases and their substrates [11–14]. Specific physical interactions between components of the cascade can also be achieved by scaffold proteins. Scaffold proteins, such as KSR-1 and ß-arrestin, bind the MAPK, MAP2K and MAP3K of a particular cascade and bring them together to form a functional signaling complex [15–17]. The scaffold complexes may also include upstream GTPases and cytoskeletal components as well as cell surface receptors [18–20].

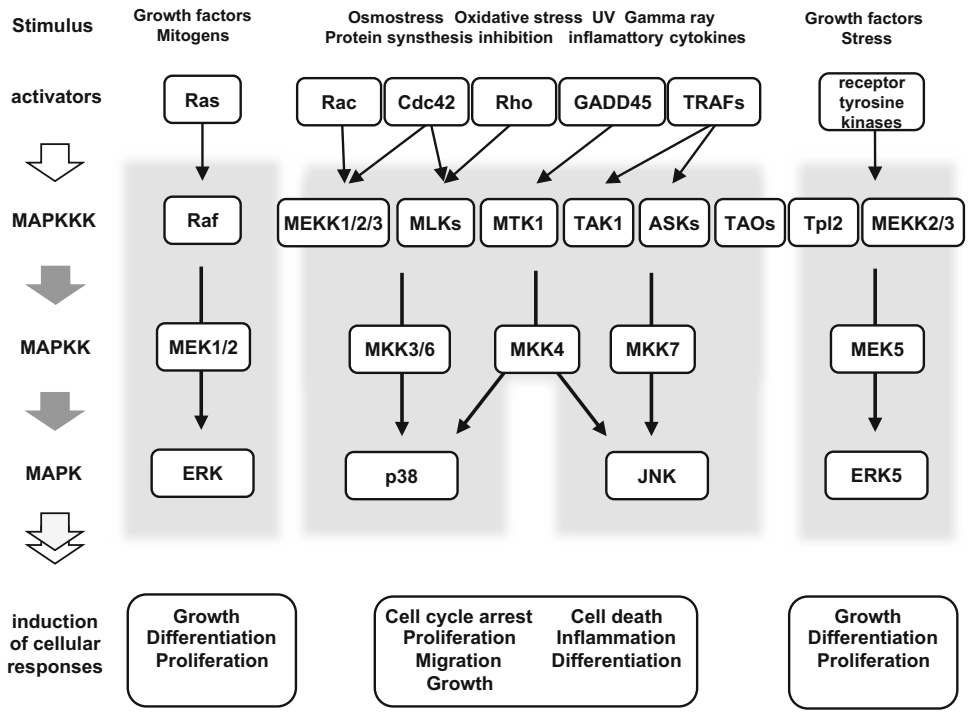

Fig. 1 Schematic diagrams of conventional MAPK signaling cascades. Mammalian cells simultaneously express several subfamilies of MAPK cascades. They include the growth-promoting extracellular-signal regulated kinase (ERK) family and the growth-suppressing stress-activated protein kinase (SAPK) families, the JNK and p38, and the relatively recently discovered ERK5. The ERK family cascade consists of a three-tired kinase cascade of a MAP3K (Raf), MAP2K (MEK) and MAPK (ERK). Activation of a small-G protein Ras triggers the activation of Raf and thus begins the cascade. Similarly, a wide variety of stresses activates the SAPK cascades via diverse stress MAP3Ks and their activators. Although there are at least 14 stress MAP3Ks, many fewer MAP2Ks (MKK3/4/6/7) and MAPKs (p38α/ß/γ/δ and JNK1/2/3) function in the stress response pathway. The activators of stress MAP3Ks include the small-G proteins (Rac, Rho and Cdc42), TRAFs and GADD45s. The MEK5-ERK5 pathway is also activated by several types of stresses and growth factors and shares some stress MAP3Ks (MEKK2/3) with JNK and p38. *Arrows* indicate the activation signal. Abbreviations used in this figure are as follows: *MEK* MAPK/ERK kinase, *MEKK* MEK kinase, *MLK* mixed-lineage kinase, *TAK1* transforming growth factor ß-activated kinase, *MTK1* MAP three kinase 1, *ASK* apoptosis signal-regulating kinase, *TAO* thousand and one amino-acid kinase, *Tpl* tumor progression locus, *TRAF* tumor necrosis factor receptor-associated factor, *GADD* growth arrest and DNA damage-inducible protein

Visualization of signal transduction

Monitoring the dynamics of signaling molecules using fluorescent proteins

Rapid advances in fluorescence technology have made a new trend in biological studies toward analysis of the behavior of a molecule in live cells, tissues and organisms under physiologically relevant conditions instead of analysis using fixed samples. In particular, the use and application of fluorescent proteins (FPs) have been significantly improved in recent years so that FPs are now indispensable tools to study intracellular signal transduction. We will therefore first describe FP-based imaging methods.

Signaling molecules often change their properties upon activation, including changes in subcellular localization, protein-protein interaction (or interaction with other molecules such as DNA or RNA) and protein conformation. In addition, changes in the status of post-transcriptional modifications (such as phosphorylation, acetylation, glycosylation or ubiquitination) are also frequent events in signal activation. For example, ERK resides in the cytoplasm in its resting state, but translocates into the nucleus upon activation by MEK. To monitor the ERK localization in cells, an approach is to exogenously express an ERK-green fluorescent protein (GFP) fusion protein in cells and to obtain time-lapse fluorescence images in real time [21, 22]. Similarly, changes in the subcellular distribution of almost any molecule can be monitored using a fairly simple method (Fig. 2a). Currently, a variety of FP colors has been developed whose range spans almost all visible wavelengths [23] as well as invisible ones near infrared [24, 25]. The wide selection of FPs allows multiplex analyses by using several differently labeled molecules simultaneously [26]. FPs with unusual photo-physical properties have also been developed. Ando and colleagues, for example, developed a reversible light-switchable FP (Dronpa) [27]. By using Dronpa, they succeeded in measuring the rates of ERK nuclear import and export separately, a difficult problem to address using previous methods. More recently,

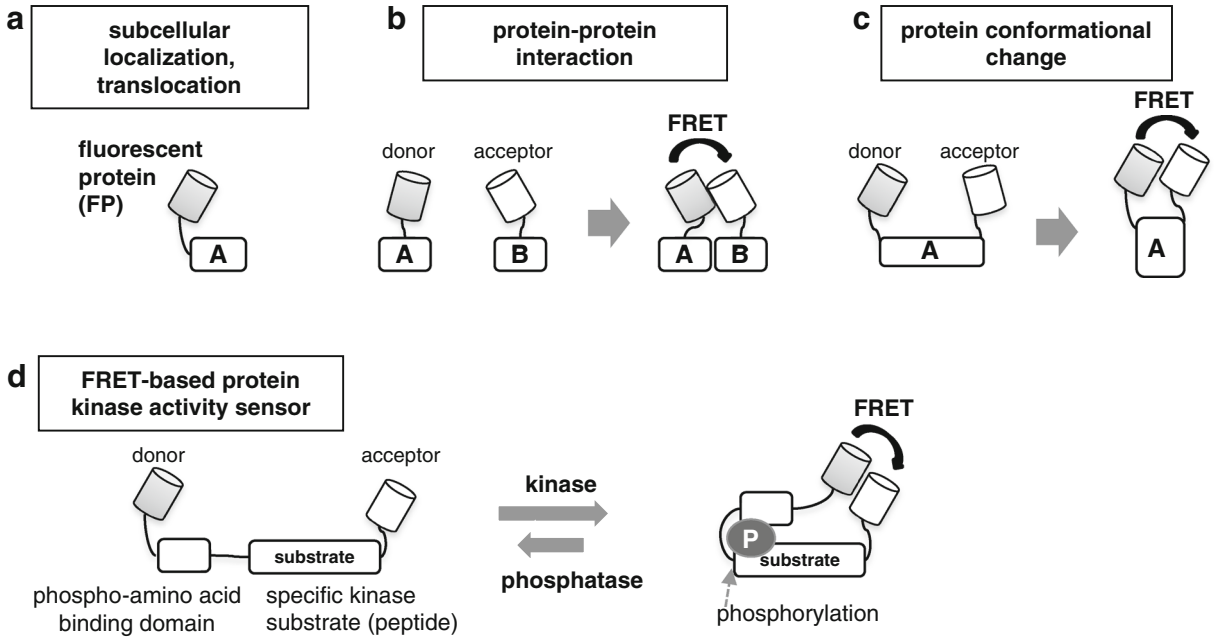

Fig. 2 FP-based analysis of cellular signaling. The behavior of a molecule in a living cell can be analyzed using fluorescent proteins (FPs). **a** A subcellular localization (or translocation) of a protein can be visualized by expressing FP-fused protein in cells. **b** The intermolecular interaction can be optically measured as changes in the FRET efficiency between two fused FPs, respectively, to the two target proteins. **c** The intramolecular protein conformational change can be detected by measuring the FRET efficiency between two FPs fused to the same target protein. **d** Measurement of protein kinase activity by a FRET-based sensor. The sensor includes a phosphorylation site specific to the kinase and the phospho-amino acid binding domain as well as two FPs located, respectively, at the N and C termini. Phosphorylation of the substrate region by an endogenous kinase allows it to bind to the phospho-amino acid binding domain within the sensor, and the resulting intramolecular conformational change between two FPs can be detected as an increase in FRET efficiency. Similarly, other post-translational modifications, such as glycosylation, methylation and ubiquitination, can also be visualized by including a specific binding domain for the modified peptide in place of the phospho-amino acid binding domain

super-resolution imaging techniques using the light-switchable FP have been developed, and the techniques permit us to achieve image resolution at ~20 nm beyond the diffraction limit [28]. The invention and use of novel FPs [29] have therefore enabled much deeper understanding of how intracellular molecules are dynamically regulated in a cell.

FP-based FRET imaging

Signaling events are often accompanied by intermolecular interactions. Thus, methods have been devised based on the Förster resonance energy transfer (FRET) to determine how protein-protein interactions occur in situ [30]. FRET is a phenomenon in which an excited donor fluorophore transmits its energy to a neighboring acceptor fluorophore without irradiative emission, thereby inducing fluorescence emission from the acceptor fluorophore. Therefore, FRET allows optical measurement of changes in the distance between two molecules. For example, when cyan FP and yellow FP are positioned close together, the light-excited cyan FP (donor), instead of emitting blue radiation, transfers the energy to the yellow FP (acceptor), which then emits yellow light. The efficiency of FRET is mainly determined by three factors under the given experimental conditions: (1) the distance between the donor and acceptor, (2) the orientation factor, which is determined by the directions of the emission transition dipole of the donor and the absorption transition dipole of the acceptor, and (3) the spectral overlap of emission and excitation wavelengths of the two fluorophores (see review [30] for details). By expressing FP-fused target proteins in living cells, changes in the distances (and orientations) of the target molecules can be optically measured as a change in FRET efficiency (Fig. 2b). Importantly, FRET efficiency is inversely proportional to the sixth power of the distance and highly sensitive to distance changes in the range of 1–10 nm. Since this distance range is also suitable for detecting conformational changes within proteins, a change in the intramolecular conformation can be detected by FRET analysis by fusing both donor and acceptor FPs to the same target protein (Fig. 2c).

Monitoring the endogenous activity of the signaling molecules using FRET biosensors

Sophisticated FRET-based biosensors have been developed that allow detection of endogenous activities of signaling

molecules. Biosensors for Ca^{2+} [31], PKA [32] and small GTPases [33, 34] were some of the earliest sensors to be developed. Small GTPase sensors have been useful for analysis of MAPK activation. The active GTP-bound form of the small GTPase Ras binds to and activates the MAP3K Raf. Thus, activation of Ras can be detected by monitoring the Ras-Raf interaction. In 2001, Mochizuki and colleagues developed a single-molecule FRET sensor for Ras activation that consists of yellow FP, Ras, Raf and cyan FP connected in series with optimized amino acid linkers. Using this sensor, they succeeded in visualizing growth factor-dependent Ras activation in living cells [33]. Soon thereafter, biosensors for endogenous kinase activity were reported [35]. Kinase activity sensors in general include a phosphorylation site specific to the kinase linked in tandem to the phospho-amino acid binding domain and two FPs (cyan and yellow) at the N and C termini, respectively (Fig. 2d). Phosphorylation of the substrate site by endogenous kinase allows it to bind to the phospho-amino acid binding domain in the sensor, which leads to a closer association between the FPs. Thus, endogenous kinase activity can be measured as an increase in FRET efficiency. Kinase activity sensors for ERK (i.e., ERKus [36], EKAR [37] and ERKy [38]) and JNK (i.e., dJUN-FRET [39], JNKAR1 [40] and JuCKY [41]) have been reported. A reporter for stress-responsive MAP3K (SAP3K) was also developed in a similar manner, but it included a modification to the general kinase reporter [42]. In the SAP3 K reporter, the C-lobe region of a MAP2K (MKK6) was used instead of the short substrate peptide in the MAPK reporter because inclusion of the MAP3K-specific docking site in the C-lobe region of MAP2K increases the efficiency and specificity of MAP3K-dependent phosphorylation [9]. Representative FRET-based observations on MAPKs and their related signaling are summarized in Table 1.

Advanced methods for the generation and use of FRET sensors

Development of a FRET sensor is a laborious process since even a single amino-acid substitution might greatly affect its properties because of the high sensitivity of the FRET efficiency to the distance between the FPs and their relative orientation. To mitigate this difficulty, methods have been reported that rationally design FRET sensors or optimize their sensitivity. These include modification of the FPs themselves [43–47] and optimization of the arrangement of domains and linker regions in between the FPs [48–50]. An increasing number of FP-based FRET reporters now allow detection of a variety of molecular activities as well as a variety of environmental factors such as membrane potential [51–53], pH [54], temperature [55] and Redox states [56–59]. For the list of recently developed sensors, see the comprehensive reviews [60, 61].

To determine how cells recognize and properly adapt to their surrounding environment, multiparametric analysis will be helpful for understanding how environmental information is transduced from one molecule to another. For this, several methods have also been developed that allow simultaneous imaging of one FRET sensor together with a second FRET sensor [62–65] (for a review, see [66]).

It should be noted that the FRET signal might result in false negatives attributed to the inadequate orientation of the FPs even when the two FP-fused proteins are interacting and the donor and acceptor are in close proximity. Conversely, intrinsic weak affinity of FPs for each other may cause a false-positive FRET signal especially when the effective concentration of the sensor is increased, for example, by anchoring the sensor to the microdomain of the plasma membrane [43]. The FP fused protein becomes substantially larger than the corresponding endogenous protein, and the attached FP might interrupt naturally occurring protein-protein interactions. As for any other kind of sensor, it is important to confirm that the sensor selectively and accurately reports the status of endogenous protein activity. It should be additionally emphasized that the observed signal results from mutual interactions between the sensor and the endogenous signaling molecules, hence suffer from the artifact attributed to the expression of the sensor by itself. For example, the intracellular signaling might be perturbed by the stoichiometric changes due to overexpression of the sensor.

The advantages of FP-based FRET over other reporters (i.e., chemical fluorescent dyes) are that these genetically encoded sensors can be easily transferred into target cells by means of virus infection, DNA (RNA) transfection or electroporation not only in cultured cells, but also in intact animal cells, and that their expression can be precisely controlled using tissue-specific or inducible promoters.

Spatial regulation of MAPK

Stimulus-specific distinction in the MAPK activation locus

Important questions in MAPK signaling are how MAPKs discriminate between types of stimuli and how they induce a proper adaptive response. FRET studies have revealed the importance of the localization of signaling proteins to appropriate subcellular compartments for their function. Uhlik and colleagues analyzed how intracellular p38 signaling is initiated in cells during hyperosmotic sorbitol stimulation [67]. For this, they explored protein-protein

Table 1 Summary of representative FRET imaging for MAPKs and related signaling

Target	FRET sensor	Sensor type	Stimulation/application (Cells)	Author	Year	References
MEKK3, OSM (scaffold)	–	Intermolecular FRET	Osmostress (Cos-7)	Uhlik et al.	2003	[67]
SAP3Ks	SAP3K reporter	Kinase activity sensor	Osmostress, DNA damage, UV-C, TNF-α (Cos-7, Hela)	Tomida et al.	2009	[42]
Ras, Rap1	Raichu-Ras, Raichu-Rap1	Intramolecular FRET	EGF, NGF (Cos-1, PC12)	Mochizuki et al.	2001	[33]
Rac, Cdc42	Raichu-Rac, Raichu-Cdc42	Intramolecular FRET	Cell migration (HT1080)	Itoh et al.	2002	[34]
c-Raf	Prin-c-Raf	Intramolecular FRET	EGF (Cos-1)	Terai et al.	2005	[68]
ERK	ERKus	Kinase activity sensor	EGF (MCF-7)	Sato et al.	2007	[36]
ERK	EKAR	Kinase activity sensor	Electrically evoked action potentials (hippocampal neuron)	Harvey et al.	2008	[37]
ERK (*C. elegans* MPK-1)	ERKy	Kinase activity sensor	In vivo imaging, NaCl-off stimulation (*C. elegans* sensory neuron)	Tomida et al.	2012	[38]
ERK, JNK	EKAR-EV, JNKAR1-EV	Kinase activity sensor	EGF (Hela)	Komatsu et al.	2011	[48]
ERK	EKAR-EV	Kinase activity sensor	EGF (MCF-10A)	Albeck et al.	2013	[104]
ERK	EKAREV, -NLS	Kinase activity sensor	In vivo imaging, laser ablation (mouse auricular epidermis/dermis)	Kamioka et al.	2012	[98]
ERK	EKAREV-NES, -NLS	Kinase activity sensor	In vivo imaging (mouse mammary gland epithelial cells), fetal bovine serum (MDCK, NRK-52E, Hela, etc.)	Aoki et al.	2013	[105]
JNK (*Drosophila*)	dJun-FRET	kinase activity sensor	RNAi screening (Drosophila BG-2)	Bakal et al.	2008	[39]
JNK	JNKAR1	Kinase activity sensor	Anisomycin, osmostress, TNF-α (Hela)	Fosbrink et al.	2010	[40]
JNK	JuCKY	Kinase activity sensor	Anisomycin (Hela)	Suzuki et al.	2010	[41]
Ras	FRas-F	Intermolecular FRET	Electrically evoked action potentials (hippocampal neuron)	Yausda et al.	2006	[73]
Ras	FRas-F	Intermolecular FRET	2-photon glutamate uncaging (hippocampal neuron)	Harvey et al.	2008	[75]
ERK	EKARnuc	Kinase activity sensor	2-photon glutamate uncaging (hippocampal neuron)	Zhai et al.	2013	[76]

interactions of signaling components in situ by FRET imaging. They showed that MEKK3 (MAP3K) forms a complex with a novel scaffold protein (OSM) in the cytoplasm and that this complex rapidly moves to actin-containing membrane ruffles upon hyperosmotic sorbitol cellular stimulation, where Rac-MEKK3–MKK3 was activated in a series upstream of p38. This study suggested that signaling proteins can be activated only at a specific locus depending on the type of stress applied to the cell. Consistent with this idea, live-cell imaging of stress-MAP3K activity using a novel FRET reporter showed that hyperosmotic stress induced MAP3K activity mainly in the plasma membrane, whereas ultraviolet light or a ribotoxic protein synthesis inhibitor induced MAP3K activation in the cytoplasm [42]. Such regulation of activity by subcellular localization seems to be a common mechanism in MAPK activation. Terai and colleagues visualized a conformational change in c-Raf (a MAP3K of the ERK pathway) upon activation by FRET [68]. They demonstrated that cytoplasmic c-Raf was recruited to the plasma membrane upon EGF stimulation, where binding to Ras induced a conformational change in c-Raf resulting in its activation. Thus, Ras binding opens up c-Raf to expose the docking site for MEK. Differences in the accessibility of regulatory proteins such as small GTPases, kinases and phosphatases, or differences in protein concentration that are due to differential cellular localization of MAPKs, might confer a different sensitivity to MAPK modules [69]. In this way, stimulation input can take place depending on the location where the MAPK module is activated. These studies clearly pointed out that MAPK signaling can be initiated from a specific subcellular locus and that stimulus-specific distinctions in MAPK activation loci would lead to different outcomes.

Visualization of Ras-MAPK activation in neuronal microcompartments

The importance of subcellular localization of signaling has also been extensively studied in neuronal cells. ERK is involved in many forms of synaptic plasticity and has therefore been proposed to be a key determinant of learning and memory [70, 71]. Various targets of the ERK kinase activity have been discovered in neuronal plasma membranes, spines, axons and nuclei. These targets include ion channels (i.e., the Kv4.2 channel and AMPA receptor), cytoskeleton regulators (i.e., focal adhesion kinase and Rho) and transcription factors [i.e., cyclic AMP-responsive element-binding protein (CREB) and Elk-1] (for details, see a comprehensive review [72]). A major difficulty in imaging analyses of neurons are their minute size, and quantification of FRET in neuronal microcompartments has been especially challenging. Instead of using conventional fluorescence intensity-based FRET measurement, Yasuda and colleagues carried out quantitative FRET imaging of Ras activity in microcompartments of a neuron using fluorescence lifetime measurements combined with two-photon excitation laser scanning microscopy (2p-FLIM FRET) [73]. Fluorescence lifetime imaging is based on the detection of time-resolved fluorescence decay after an ultra-short pulsatile excitation of the fluorophore (for details, see this review [74]). Harvey and colleagues demonstrated that Ras signaling can spread from an LTP (long-term potentiation)-induced spine along dendrites as long as $\sim$10 μm [75]. The same group also succeeded in imaging ERK activity using 2p-FLIM FRET [37]. Zhai and colleagues further investigated the prerequisite conditions for nuclear ERK activation and found that induction of LTP in a relatively small number of dendritic spines is sufficient to maintain nuclear ERK activity as well as activation of downstream transcription factors [76]. These studies clearly demonstrated that the compartmentalized signal activation defines the extent and duration of the subsequent signaling according to the spatial parameters (i.e., length of the axon, location of the spine and distance from the nucleus). In addition to the roles of ERK in synaptic plasticity, many studies have also shown that MAPKs are involved in axonal growth, synapse development, apoptosis, degeneration and re-generation after injury [77–80] (for details, see the comprehensive reviews [81, 82]). Undoubtedly, MAPK signaling can function as a potential target in terms of medical treatment of neurological disorders (details are reviewed in [83]). Although the mechanism by which MAPK signaling contributes to those neuronal functions is still largely unknown, the increasing number of imaging tools will facilitate understanding of the dynamic nature of signal transduction in living neurons.

Temporal regulation of MAPKs

Cell fate determination by the duration of MAPK activity

Another important factor that determines MAPK signaling is timing. Many biochemical studies have pointed out that MAPK activities occur in either a transient or a sustained manner depending on the type of stimulation. One of the best known examples of this type of regulation is signaling in the neuroendocrine cell line PC12, which has distinct outcomes resulting from specific stimulation. Treatment of PC12 cells with EGF induces transient ERK activation and cell proliferation, whereas treatment with NGF causes relatively persistent ERK activation and the cells differentiate into neuron-like cells [84, 85]. A similar

phenomenon has been reported for the stress MAPK pathway. TNF induces JNK in a biphasic manner with robust initial transient activation (<1 h) followed by relatively lower sustained activation (1–6 h). Ventura and colleagues hypothesized that the time course of JNK signaling may play a role in the determination of a specific outcome. They utilized a chemical genetic approach to specifically manipulate the catalytic activity of JNK at a fixed time and found that apoptotic signaling induced by JNK required sustained activation and that transient activation of JNK induced cell survival signaling [86]. Thus, the duration of MAPK activity is as important as the extent of MAPK activity in determining cellular responses.

Systems analysis of MAPK signaling based on FP imaging

Owing to recent progress in the so-called "omics" analyses, such as transcriptome, interactome and phospho-proteome analyses using mass spectroscopy and to high-throughput gene expression analysis, a tremendous number of connections between signaling molecules that are involved in their regulation have been revealed [87] (for details, see the reviews [88, 89]). This information has forced a revision of our understanding of signal transduction, suggesting that it would be more appropriate to consider that MAPKs function as part of a large signaling network that encompasses signaling of the entire cell rather than a simple straightforward modular kinase cascade. To date, mechanisms and regulation of MAPK activation have been elucidated in detail, and it is becoming possible to predict the dynamics of MAPK activation in silico [90–93]. However, explanations of MAPK dynamics that occur in actual cells under physiologically relevant conditions have not yet been elucidated.

Identification of the core regulatory mechanism of MAPK dynamics in living cells was first successfully addressed using yeast cells. Mettetal and colleagues applied a system identification method combined with real-time imaging to analyze the temporal regulation of yeast osmo-responding Hog1 MAPK signaling [94]. They stimulated the cell with repeated salt shock pulses and simultaneously measured the kinetics of nuclear translocation (activation) of a Hog1-FP fusion protein. By analysis of the stimulation-frequency dependence of the Hog1 response, the authors succeeded in identifying regulatory negative feedback loops in yeast osmo-adaptive signaling. Using similar methods, Hersen and colleagues explored the time scales of signal activation and inactivation, and they demonstrated that the kinetics of Hog1 signaling are distinctly regulated depending on the type of upstream osmolality-sensing receptors [95]. The combined application of live-cell imaging and systems analysis thus allows a knowledge of the functional topology of a signaling network. Recently, transgenic animals harboring a FRET sensor have been generated allowing visualization of signaling events in situ under physiologically relevant conditions [96–98]. Tomida and colleagues, using a combination of in vivo FRET imaging with a systems-analysis method (Fig. 3), determined how information in the external cellular environment that stimulates cells is transduced in the cell of a living animal, using the nematode *Caenorhabditis elegans* as a model system [38]. In that study, a FRET-based ERK-family kinase sensor was created and expressed in a sensory neuron, and the nematode was then exposed to various cyclic patterns of repetitive salt stimulation in a flow chamber. This neuronal MAPK imaging analysis demonstrated that the intensity and duration of MAPK activity are determined by the temporal pattern of input stimulation, i.e., a combination of stimulation period length, stimulation pulse length and pulse frequency. The highest MAPK response was achieved following a stimulation of modest frequency,

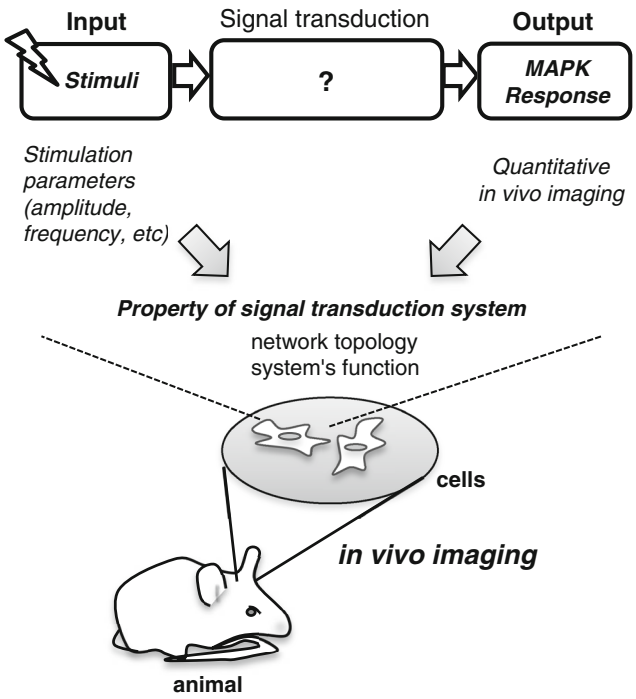

Fig. 3 Systems analysis of signal transduction in vivo. A systems-analytical method can be combined with the in vivo imaging to study the regulation and function of the network of the signaling molecules in situ. The analysis typically consists of a defined set of input stimulations (for example, the cyclic pulsatile salt stimulation, whose parameters, such as the amplitude, frequency and duration of the stimulation pulses are varied) followed by measurement of output responses (i.e., MAPK activity) from which the system properties are deduced. An in vivo imaging of the signaling inside the target cell can be achieved using a genetically encoded sensor, which can be expressed by standard gene transferring methods (i.e., viral infection and DNA transfection)

whereas a much higher frequency or less frequent stimuli resulted in transient or low-level MAPK activation. Identification of such a nonlinear relationship in which the resulting MAPK activity is not determined by the total amount of stimulation but by the adequate timing of cyclic stimulations also provided a hint regarding its regulatory mechanism. Further in vivo Ca^{2+} imaging analysis using this system demonstrated that a similar nonlinear Ca^{2+} signaling response determined the temporal MAPK dynamics in the nematode neuron [38]. These studies pointed out that an endogenous signaling system can properly respond to the outer fluctuating environment by interpreting the temporal information (i.e., frequency) of the environmental change and adequately inducing MAPK activation according to the temporal parameter.

Oscillatory MAPK activation

Recent imaging analysis further demonstrated another characteristic behavior of MAPKs. Theoretical studies of MAPK dynamics have predicted the existence of oscillatory kinase activation [99, 100]. This hypothesis was proven by studies that focused on feedback regulation of MAPKs in mammalian and yeast cells [101–105, 108]. Nakayama and colleagues investigated FGF-induced oscillatory Hes1 expression and ERK activation and found that a negative feedback phosphorylation of the guanine nucleotide exchange factor Sos (which activates Ras) by ERK [106] is required for this oscillation [101]. Shankaran and colleagues also demonstrated that EGF stimulation induces oscillatory ERK translocation between the cytosol and nucleus. They quantified temporal parameters of ERK translocation and deduced a model that suggested the involvement of a similar negative feedback loop by ERK [103]. Indeed, typical biochemical oscillators often involve a negative feedback loop with a time delay (for a review, see [107]). Albeck and colleagues [104] demonstrated that the frequency of the EGF-stimulated ERK oscillation played important roles in the regulation of cellular proliferation. Interestingly, Aoki and colleagues described another type of ERK activity oscillation that can be stochastically induced by periodic activation of Raf and that occurs under normal growth conditions. Although the detailed mechanism of how Raf is stochastically activated remains elusive, they clearly demonstrated that cellular density regulates the frequency of the stochastic ERK oscillation and, moreover, that oscillatory ERK activities induce cell proliferation signals [105]. Recently, Regot and colleagues developed the novel kinase translocation reporters (KTRs) that translocate into the nucleus upon phosphorylation by ERK, JNK and p38 MAPKs, respectively. Using these reporters, they demonstrated that ERK and JNK activities fluctuated in living cells. They further conducted multiplexed imaging analysis using KTR and other reporters that were fused to different FPs and examined the correlation and the crosstalk between different signaling pathways. Interestingly, they found that not the peak amplitude but the peak number of oscillatory ERK activities was modulated by the p38 activity [108]. Importantly, oscillatory MAPK dynamics have been difficult to address without single-cell measurement because averaging cell population responses will negate stochastic or asynchronous oscillation occurring in individual cells. Surprisingly, oscillatory activation of MAPK has also been found in the yeast Fus3 pathway [102], suggesting that some conserved regulatory mechanism may underlie the oscillatory dynamics of MAPK signaling.

Identification of temporal codes in signal transduction

As described above, analysis of the temporal regulation of MAPK signaling has suggested a universal mode of signal transduction that likely utilizes the temporal parameter as a code. The regulatory mechanisms and significance of such temporal codes of MAPK signaling under physiological circumstances are still under investigation. However, lessons from studies on other signaling systems that exhibit similar temporal behaviors, such as NF-kB, p53 and PKC signaling, would provide clues for such studies [109–112]. For example, oscillatory NF-kB determines the set of cytokine genes to be expressed according to the frequency of NF-kB activation spikes [109, 113]. When considering fluctuations in extracellular environmental signals under physiological conditions, it would be important to discriminate physiologically relevant information from surrounding noises. In this sense, temporally determined signaling (such as the frequency modulation) may be advantageous in terms of a gain in robustness against environmental noise.

Concluding remarks and perspectives

MAPK signaling functions to relay information from the outer environment in order to induce distinct cellular responses. To properly perceive and respond to fluctuating environmental changes, the signal should filter out noises, amplify physiologically relevant information, coordinate information with other incoming signals and carry this information to the proper subcellular locus where targeted molecules execute proper effector functions depending on the cellular context. As described above, recent successful quantification of molecular states under physiologically relevant conditions has revealed the roles of signaling molecules in terms of time and space in the determination of cell fates (Fig. 4). Such spatially and temporally regulated signaling would be beneficial to the cell because variable targets could be simultaneously but

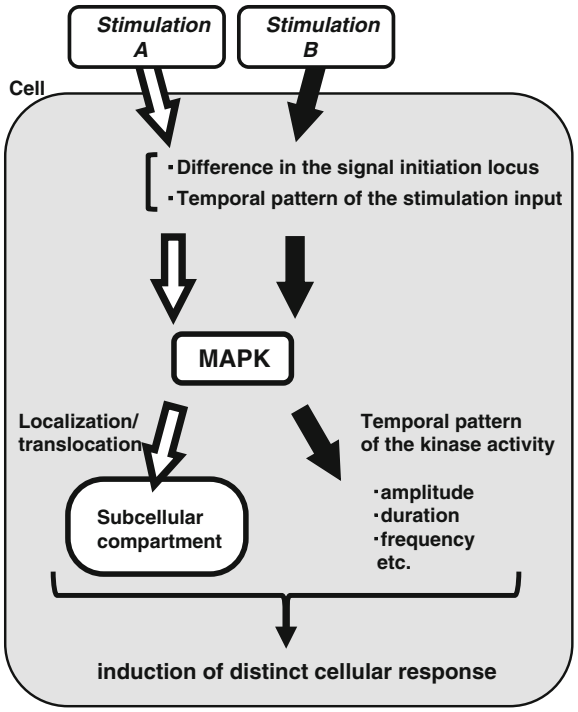

Fig. 4 Schematic illustration of the role of dynamic MAPK signaling. The information of the outer environment (stimulation or input) can be transduced into the inside of the cells, where the network of signaling molecules transmits this information to activate the MAPK molecule. The type and location of the input stimulation, as well as its strength, duration and temporal pattern, determine the subsequent dynamics of MAPK activity. MAPK can be activated either at a specific locus or in the entire cytoplasmic region, or translocated into a specific compartment (i.e., the nucleus), to execute a proper effector function such as gene expression. Furthermore, MAPK activation can be transient, long sustained or oscillatory in manner. Subsequent cellular function can be regulated depending on the temporal parameters (i.e., oscillation frequency) of MAPK activation. Thus, a combination of a set of the spatial and temporal parameters of MAPK dynamics encodes the cellular context to induce a specific adaptive response

specifically regulated by a limited number of MAPK subtypes. Furthermore, these signals could be processed or filtered so that only physiologically relevant information is transduced. Abnormal regulation of MAPK signaling is responsible for human diseases such as cancer, autoimmune defects, neurological disorders and metabolic diseases; hence, MAPK signals are of potential importance for the diagnosis and development of therapeutic drugs. It will be beneficial to determine whether the misregulation of the spatio-temporal dynamics of the signaling is involved in various diseases and disorders using animal models. Although there have been only a few examples of in vivo signaling dynamics described to date, future advances in imaging techniques and analytical methods will accelerate the progress of research of the dynamics of the signaling within cells or within intact organs or animals.

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