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Reviews and Mini-reviews

Multiplex live imaging approaches to interrogate the interplay of multiple signaling pathways

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Abstract

Multiplex live imaging enables simultaneous visualization of multiple signaling pathways in living cells, offering real-time insights into complex cellular networks. This methodology is essential in research fields such as cancer biology, where signaling activities exhibit heterogeneity, feedback regulation, crosstalk, and dynamic changes during pathological progression and the acquisition of therapeutic resistance. While conventional biochemical assays advanced our understanding of signaling signatures through static or population-level analyses, they lack the temporal resolution required to capture dynamic events at single-cell resolution.

Recent methodological innovations have expanded multiplex live imaging through several strategies. Spectral multiplexing exploits broadened fluorescent protein palettes and optimized biosensor combinations, sometimes coupled with intracellular multiplexing methods that distinguish signals by targeting fluorescence to subcellular compartments. Intercellular multiplexing distributes reporters across cell populations, and temporal multiplexing leverages optical switching to separate signals over time. Additional modalities such as fluorescence anisotropy, fluorescence lifetime, and

Raman imaging provide orthogonal readouts. Furthermore, computational approaches reinforce multiplex strategies by improved spectral unmixing, often complemented by deep learning-based algorithms. Collectively, these advances enable simultaneous tracking of multiple signaling pathways within single cells, revealing how diverse inputs are integrated into cellular responses.

Here we review current strategies for multiplex live imaging, especially highlighting its applications to cancer signaling networks. Progress in fluorescent biosensor development, imaging technologies, and computational analysis will further promote the exploration of dynamic cellular regulations in basic research and translational medicine.

Running head

Multiplex live imaging approaches

Keywords

multiplex live imaging, fluorescent biosensors, signal dynamics, image analysis, cancer heterogeneity

Introduction

Cellular signaling pathways are fundamental to a wide variety of physiological and pathological processes, including cell proliferation, differentiation, apoptosis, and responses to environmental stimuli. In contrast to methods on fixed or lysed cells such as Western blotting, immunostaining, and Enzyme-Linked Immunosorbent Assay (ELISA), live imaging allows tracking of signal activities in real time typically by employing (1) fluorescent dyes, (2) genetically encoded fluorescent proteins, or (3) spectroscopic properties such as Raman shifts. Fluorescent dyes are useful in visualizing the target without the need for genetic manipulation. Genetically encoded fluorescent proteins are represented by Förster resonance energy transfer (FRET)-based biosensors, which allow for stable tracking of signaling activities. Unlike these fluorescence-based labeling strategies, spectroscopic approaches provide label-free visualization by relying on the intrinsic optical properties of biological molecules. All of these strategies have been refined for greater sensitivity and specificity (Fujita & Urano, 2024; Terai et al., 2019; Togo et al., 2025).

Despite the advantages of live imaging, a major limitation lies in the simultaneous visualization of multiple signaling activities within individual cells (multiplex live imaging). This limitation primarily stems from the finite range of the fluorescence spectrum and the substantial spectral overlap among commonly used fluorescent proteins. Most conventional live imaging systems allow observation of two to three fluorescent proteins within minimal crosstalk, which is often insufficient to capture the complexity of signaling networks. For instance, concurrent activation of Extracellular signal-regulated kinase (ERK) and Phosphatidylinositol-3 kinase (PI3K) pathways, or the dynamic interplay between calcium and cyclic AMP is crucial for understanding how cells integrate diverse extracellular cues into coherent cellular responses (Mendoza et al., 2011; Willoughby & Cooper, 2007).

Recently, several novel strategies for multiplex live imaging have been developed through advances in biosensor engineering, microscopy technologies, and computational analyses. For example, orthogonal biosensors among FRET-based biosensors, intensity-based fluorescent reporters, and kinase translocation reporters (KTRs) enable multiple readouts in single cells. Advances in microscopy have extended the detectable fluorescence spectrum with improved sensitivity. Computational approaches such as spectral unmixing and machine learning-based signal deconvolution further enhance the multiplex capability by extracting distinct signals from overlapping spectra. In this review, we summarize strategies to achieve multiplex live imaging and highlight recent studies that showcase representative multiplex biosensors as well as advanced image acquisition and analysis approaches. Furthermore, to highlight the

significance of multiplex imaging in a specific research field, we introduce its applications and prospects in cancer research.

Because of the inherent limitation imposed by the finite spectral range available for fluorescence live imaging, multiplex live imaging can only be achieved by efficiently utilizing this limited spectral range or by compromising certain features such as spatial resolution and the dynamic range of individual biosensors. Hereafter, we introduce three major strategies for multiplex live imaging (Figure. 1): (1) spectral multiplexing, which maximizes the use of the finite spectral range by employing fluorescent biosensors with minimal spectral overlap or by incorporating ultraviolet or near-infrared fluorescent proteins; (2) spatial multiplexing, which is achieved by targeting different fluorescent biosensors to distinct subcellular locations of identical cells (intracellular multiplexing) or distinct cells in identical cell populations (intercellular multiplexing); (3) temporal multiplexing, which alternates the type of fluorescent biosensors over time to sequentially monitor multiple targets or utilizes time-dependent photophysical properties such as lifetime. Each of these three strategies offers distinct advantages and trade-offs, underscoring the importance of selecting an approach best suited to the specific research objectives and available experimental resources. Following these fluorescence-based strategies, we will briefly review spectroscopic imaging approaches and computational approaches that can further extend the capabilities of multiplex imaging.

1. Spectral Multiplexing

1.1 Fluorescent proteins for multiplex imaging

A straightforward way to achieve multiplex live imaging is to employ multiple fluorescent biosensors with distinct excitation and emission spectra (spectral multiplexing). A wide palette of fluorescent proteins has become available through the development of fluorescent protein variants, including Blue Fluorescent Protein (BFP), Cyan Fluorescent Protein (CFP), Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), Kusabira Orange (KO), Red Fluorescent Protein (RFP), and Infrared Fluorescent Protein (iRFP) (Chudakov et al., 2010; Day & Davidson, 2009; Lukyanov et al., 2010) (Figure. 2A). “Enhanced” fluorescent protein variants such as Enhanced Blue Fluorescent Protein (EBFP), Enhanced Cyan Fluorescent Protein (ECFP) and Enhanced Green Fluorescent Protein (EGFP) show brighter signals and monomeric variants such as monomeric Kusabira Orange kappa (mKOκ), monomeric Cherry (mCherry), mScarlet and monomeric Kate (mKate) facilitate fluorescent tagging to the target molecules by avoiding aggregation of the fluorescent proteins.

This strategy of using multiple fluorescent proteins can be extended by employing fluorescent proteins at spectral extremities (i.e., ultraviolet and far-red fluorescent proteins). Ultraviolet and blue fluorescent proteins such as Sirius (Tomosugi et al., 2009) and EBFP (T.-T. Yang et al., 1998) represent the shorter-wavelength end of the fluorescent protein spectrum. Although these fluorescent proteins are not preferably used for single-color imaging due to high cellular autofluorescence and phototoxicity, they expand the available spectrum window for multiplex imaging. In contrast, red and far-red fluorescent proteins are convenient choices for combination with standard fluorescent proteins (e.g. EGFP) because of their lower autofluorescence and phototoxicity. Suitable candidates for multiplex imaging include mCherry (Shaner et al., 2004), mKate (Shcherbo et al., 2007) and mScarlet (Bindels et al., 2017), which offers high brightness and photostability (Shang et al., 2024). Representing the strategy of using multiple fluorescent proteins, Brainbow enables stochastic expression of different combinations of fluorescent proteins, thereby labeling individual neurons in more than 90 distinct colors (Livet et al., 2007). Although Brainbow is typically applied to fixed tissues, Boulina et al. extended this approach to live imaging in Drosophila using CFP, YFP and RFP (Boulina et al., 2013).

1.2 Multiplexing of fluorescent proteins in the detectable spectral range

When multiple fluorescent biosensors are used within the finite spectrum window (typically 300 – 700 nm), cross-excitation and bleed-through must be carefully minimized. Especially, for multiplexing FRET-based biosensors, minimizing spectral overlap between fluorescent proteins is crucial for reliable quantitative analysis. Watabe et al. combined CFP-YFP-based FRET biosensor with mKOκ-mKate-based FRET biosensor to simultaneously monitor Protein Kinase A (PKA) and ERK activity (Figure. 2B) (Watabe et al., 2020). Ai et al. identified mTFP1-mCitrine and mAmetrine-tdTomato as an optimal dual FRET pair after systemically examining the bleed-through among various donor-acceptor combinations (Ai et al., 2008). Niino et al. demonstrated dual FRET imaging of Ca^{2+} and cAMP using Sapphire-RFP and CFP-YFP-based FRET biosensors, combined with linear unmixing (Niino et al., 2009).

While FRET between fluorescent proteins is widely used, recent studies have shown the advantage of dye-fluorescent protein FRET systems, which offer higher flexibility in donor-acceptor pairing (Hellweg et al., 2023; Suzuki et al., 2022). Beyond emission-based separation, the excitation spectrum provides another dimension for multiplexing. Mehta et al. developed excitation ratiometric (ExRai) probes that monitor kinase activities via a single fluorescent emission excited differentially at 380 and 480 nm depending on target activity (Mehta et al., 2018). Chen et al. employed six frame-synchronized excitation lasers to differentially detect six subcellular targets (K. Chen et

al., 2021). For live imaging of kinase activity, kinase translocation reporters (KTRs) (Kudo et al., 2018; Regot et al., 2014) are advantageous because they require only a single fluorescent protein, unlike FRET-based biosensors that occupy a broader spectral range. Maryu et al. combined KTRs for ERK and AKT activities (Maryu et al., 2016). Tsai et al. achieved simultaneous live imaging of three targets (ERK, PKA, and calcium) by multiplexing tdTomato, emiRFP670, and GCaMP8s (Tsai et al., 2025). The multiplexity of the probes is ultimately limited by the spectral separation of fluorescent proteins free from significant bleed-through and cross-excitation. To overcome this spectral constraint, spectral multiplexing is often integrated with other approaches such as spatial multiplexing to further expand the number of detectable signaling activities.

2. Spatial Multiplexing

2.1 Intracellular multiplex imaging

Spatial multiplexing distinguishes fluorescence signals from different biosensors by location. Intracellular multiplex imaging exploits subcellular localizations (e.g., membrane, cytoplasm, nucleus) for the spatial segregation. High resolution images are often required to observe the subcellular components. To tag fluorescent biosensors to the nucleus or cytoplasm, nuclear localization signal (NLS) and nuclear export signal (NES) are commonly used whereas CAAX motifs are utilized to anchor biosensors to the plasma membrane. Histone H2B and lamin B1 are used to tag fluorescence to nucleus and nuclear membrane, respectively (Figure. 3A). As a tool for the identification of subcellular compartments, Imanishi et al. developed NuCyM (Nucleus, Cytosol, and Membrane) reporter, which distinctively labels the three subcellular compartments by mCherry and iRFP. They leveraged NuCyM to recapitulate hematoxylin and eosin (H&E)-like fluorescence images together with ERK activity visualization (Imanishi et al., 2018). Spatial multiplexing can be combined with spectral multiplexing to increase the number of simultaneous readouts. Piljic and Schultz observed calcium/calmodulin-dependent protein kinase IIα (CaMKII), Protein Kinase C (PKC), and Annexin A4 activity by multiplexing CFP-YFP-based FRET biosensors localized to the cytoplasm and plasma membrane, together with an mOrange-mCherry-based FRET biosensor that translocated between the plasma and nuclear membrane (Piljic & Schultz, 2008). Yang et al. achieved large-scale multiplex imaging by utilizing subcellular location barcodes (nucleus, plasma membrane, nuclear membrane, and cytosol) combined with deep learning-based unmixing (Chi et al., 2022; J.-M. Yang et al., 2021).

Spatial multiplexing is not limited to defined subcellular regions (e.g. nucleus vs. cytosol) but can also be expanded to any spatially separated regions in the cell. Linghu

et al. employed self-assembling peptides to form clusters of fluorescent biosensors, which they named Signaling Reporter Islands (SiRIs). SiRIs spatially separate otherwise spectrally overlapping reporters into resolvable puncta (Figure. 3B). This spatial multiplexing enabled simultaneous imaging of up to five signaling targets, including Ca^{2+}, cAMP, PKA, PKC, and ERK, within single cells (Linghu et al., 2020). Zhang et al. achieved spatial multiplexing by phase separation-based biosensors that cluster fluorescent signals. They developed the SPARK (Separation of Phases-based Activity Reporter of Kinase) system, in which kinase activity induces multivalent interactions between engineered motifs, driving the formation of bright punctate EGFP condensates. Using this approach, Zhang et al. demonstrated oscillatory dynamics of PKA activity and transient ERK activation during Drosophila tracheal metamorphosis (Q. Zhang et al., 2018). Erdoğan et al. extended the phase separation-based strategy to monitor AMP-activated Protein Kinase (AMPK) activity (AMPK-SPARK) and further created GCaMP-AMPK-SPARK, a dual reporter that simultaneously visualizes Ca^{2+} signaling and AMPK activity (Erdoğan et al., 2024).

The strategy of spatial multiplexing is highly compatible with other multiplexing approaches, effectively expanding the number of detectable fluorescent biosensors by leveraging distinct subcellular locations. However, image analysis can be challenging because it requires accurate segmentation of each spatial compartment. Recent advances in artificial intelligence (AI)-based image analysis hold great promise for addressing this issue. Nonetheless, biological images are highly variable depending on fluorescence intensity, cell type, and microscope settings, which limits the generalizability of a single analysis platform across diverse experimental conditions. Furthermore, care must be taken when interpreting signal activities associated with subcellular localizations because the target signal may exhibit location-dependent dynamics within the cell.

2.2 Intercellular multiplex imaging

Signal activity can vary considerably between individual cells. However, when such variability is relatively low, the signal state can be inferred from other cells cultured under the same conditions. Intercellular multiplexing is a spatial multiplexing strategy to overcome spectral limitations by expressing different biosensors in different cells within the same population, thereby capturing single-cell signal dynamics at the population level (Figure. 4). Compared to intracellular multiplexing using different subcellular compartments, each fluorescent biosensor signal is more easily distinguished because of the physical separation between cells. Typically, a single fluorescent biosensor is expressed in each cell, eliminating the need for spectral separation as required in spectral multiplexing. The fluorescent biosensor expressed in each cell is typically identified by the fluorescence spectrum or by the physical

separation of compartments such as individual wells of multi-well plates. Kuchenov et al. implemented this strategy by printing 40 distinct FRET biosensor plasmids onto each well of a 384-well plate to monitor EGFR, ERK, AKT, and Ras activity. Using this platform, termed FRET Multiparameter Imaging Platform (FMIP), they systematically profiled downstream network responses to EGFR mutations and drug treatments, revealing synergistic or antagonistic effects (Kuchenov et al., 2016). Kaufmann et al. combined the intercellular multiplex strategy with intracellular multiplexing by using three subcellular compartment tags (cytosol, nucleus, and peroxisomes) together with four fluorescent proteins (BFP, CFP, GFP, and YFP), generating up to 12 distinct spatial barcodes. They applied this approach in A375 melanoma cells to construct a "Signalome" of 12 key cancer-related signaling pathways including Mitogen-activated Protein Kinases (MAPKs), AKT, tumor protein 53 (p53), Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), and Wingless/Integrated (WNT) signal pathways and revealed the cell size-dependent variations in signal activity (Kaufman et al., 2022).

The major advantage of the intercellular multiplexing approach lies in the unambiguous identification of the fluorescent biosensor in each cell or cell population. This feature is especially helpful when a wide spectrum is occupied by a single fluorescence reporter, as in FRET-based biosensors. However, because different cells are used to monitor distinct target molecules, intercellular multiplexing inherently lacks the ability to examine temporal synchronicity among multiple signaling pathways in the same cell.

3. Temporal Multiplexing

3.1 Sequential acquisitions of multiple target images

Whereas spatial multiplexing separates fluorescence signals by location, temporal multiplexing separates them by temporal features such as timings of image acquisitions and time-dependent photophysical properties. In the former strategy, fluorescent biosensors are sequentially activated or recorded so that each time frame corresponds to one biosensor. Fast-scanning microscopy, such as light-sheet microscopy and spinning disk confocal microscopy, are typically employed to capture signal activities at "near-simultaneous" time points.

Photochromic Fluorescent Proteins (PFPs) are often used for temporal multiplexing because of their switchable optical properties that depend on excitation wavelength. In photochromic FRET (pcFRET) biosensor, a PFP serves as the donor fluorophore. Roebroek et al. developed Reversibly Switchable A-kinase Activity Reporter with EV-

linker (rsAKARev), a revised PKA FRET biosensor in which a photochromic fluorescent protein, mTFP0.7, functions as the donor and a non-photochromic protein, cpVenus172 serves as the acceptor. They co-expressed rsAKARev with EKAREV, a non-photochromic FRET biosensor based on ECFP and Yellow Fluorescent Protein for Energy Transfer (YPet), to monitor both PKA and ERK activity in single cells with computational signal separation. They further demonstrated that this strategy can be extended to multiplex imaging of three targets (Ca^{2+}, PKA, and ERK) within single cells (Roebroek et al., 2021). Some PFPs, such as Dronpa and Reversibly Switchable Enhanced Green Fluorescent Protein (rsEGFP), are Reversibly Switchable Fluorescent Proteins (RSFPs), which can be toggled reversibly between fluorescent 'on' and 'off' states. Willig et al. imaged EGFP- and rsEGFP2-labeled mouse cortex before and after photoactivation of rsEGFP2, and obtained rsEGFP2-specific signals by subtracting the EGFP component from the combined fluorescence signal (Figure. 5A) (Willig et al., 2021).

3.2 Multiplexing by time-dependent photophysical properties

The other strategy of temporal multiplexing is to focus on time-dependent photophysical properties. Fluorescence lifetime represents a key parameter for this multiplexing strategy and is defined as the average duration of a fluorescent molecule that remains in its excited state before returning to the ground state. Time-Correlated Single-Photon Counting Fluorescence Lifetime Imaging Microscopy (TCSPC-FLIM) enables quantitative and high-resolution measurements of fluorescence lifetimes, thereby allowing multiplex detection of spectrally overlapping biosensors and functional readouts such as FRET efficiency (Poudel et al., 2020). For instance, Damayanti et al. developed phosphorylation-sensitive biosensors for Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) and AKT that exhibit lifetime shifts upon activation, permitting simultaneous detection even in the presence of spectral overlap (Damayanti et al., 2017). Tan et al. developed a series of fluorescent proteins with distinct lifetimes, enabling multiplex live imaging of nine fluorescent biosensors through the combination of spectral and lifetime multiplexing approaches (Tan et al., 2025).

Qian et al. introduced a multiplexing approach termed TMI (Temporally Multiplexed Imaging), in which five distinct RSFPs were simultaneously expressed and unmixed based on their fluorescence decay kinetics (Figure. 5B). Using TMI, they achieved simultaneous observation of multiple kinase activities (c-Jun N-terminal kinase (JNK), ERK, p38 mitogen-activated protein kinase, and PKA) together with cell-cycle phase monitoring (Qian et al., 2023).

This strategy of sequential acquisitions of multiple target images is a relatively new approach for multiplex imaging and can also be combined with spatial multiplexing. Indeed, Qian et al. incorporated subcellular localization tags into their TMI approach to further enhance multiplexing capacity. However, temporal multiplexing inevitably comes at the cost of temporal resolution. For instance, each image acquisition cycle in TMI requires a few seconds, which may limit the detection of rapidly oscillating signals such as Ca^{2+} dynamics. Depending on the nature of the signal dynamics, ranging from fast Ca^{2+} oscillations to slow cell cycle transitions, it is critical to select the multiplexing approach (spectral, spatial, temporal, or hybrid) that best suits the experimental objectives.

Multiplexing Using Signals Other than Fluorescence Intensity

While the spectral, spatial, and temporal separation of fluorescent signals forms the basis of multiplex imaging, alternative strategies that exploit physical properties beyond fluorescence intensity provide a complementary dimension for multiplex imaging. These approaches leverage photophysical characteristics such as fluorescence anisotropy and Raman vibrational signatures, enabling the simultaneous observation of multiple fluorescent proteins that are spectrally overlapped.

Fluorescence anisotropy refers to the polarization of emitted light relative to the polarized excitation light. When a fluorescent protein is excited with polarized light, its emission remains partially polarized if the molecule remains relatively stationary during the fluorescence lifetime. However, if rotational motion occurs, for instance during molecular binding or interaction, the emitted light becomes depolarized, resulting in a decrease in anisotropy. Fluorescence anisotropy can thus be used to detect FRET efficiency as a reduction in anisotropy within donor-acceptor pairs of the same emission spectrum (homo-FRET) (Ghosh et al., 2012). Ross et al. developed Fluorescence Anisotropy Reporters (FLAREs), which detect kinase activity by ratiometric analysis of anisotropy and enable the simultaneous detection of PKA, ERK, and Ca^{2+} dynamics using a single-color fluorescent protein as the FRET readout for each target (Ross et al., 2018).

Raman spectroscopy is a vibrational spectroscopic technique that provides molecular information by analyzing energy shifts in scattered light following laser excitation (Y. Zhang et al., 2010). In contrast to fluorescence, which involves broad emission bands, Raman scattering produces spectral peaks that are approximately 50 – 100 times narrower. This high spectral resolution greatly enhances its potential for multiplexing. Fujioka et al. employed electronic pre-resonance stimulated Raman

scattering (EPR-SRS) to simultaneously visualize the enzymatic activities of β-galactosidase, γ-glutamyl transpeptidase, and dipeptidyl peptidase-4 in real time (Fujioka et al., 2023).

Imaging methods based on these properties can be combined with one another and with spatial multiplexing techniques. For instance, with the development of Raman probes capable of targeting different subcellular regions, it is now possible to spatially distinguish between multiple Raman probes (Shen et al., 2021), further enhancing multiplexing capabilities. However, these advanced techniques often require specialized instrumentation and complex experimental procedures, which may limit their accessibility in conventional laboratory settings.

Computational Approaches to Complement Multiplex Imaging

Computational approaches for multiplex live imaging have been utilized well before the recent surge in artificial intelligence (AI) or deep learning (DL)-based methodologies. Linear unmixing, a classical computational strategy, relies on reference spectra of individual fluorescent proteins to solve linear unmixing equations and separate overlapping signals. However, technical problems inherent to biological imaging, including image noise, cell-to-cell signal variability, and photobleaching, impose significant limitations on such computational techniques (Garini et al., 2006; Zimmermann et al., 2003).

Nevertheless, advanced algorithmic methods continue to be proposed to overcome these technical limitations. These include matrix factorization techniques (e.g., non-negative matrix factorization) for decomposing mixed signals, clustering algorithms for grouping pixel spectra, and the integration of deep learning frameworks for robust signal separation under noisy, photon-limited live-cell imaging conditions (Acuña-Rodriguez et al., 2022).

Recently, AI and DL have emerged as powerful strategies to enhance multiplexing performance. Neural networks improve multiplex imaging by mitigating image noise, artifacts, and spectral overlap (Pylvänäinen et al., 2023). For instance, Tian et al. applied deep learning-based lifetime phasor classification, consolidating the role of AI in discriminating diverse imaging readouts (Tian et al., 2021).

Importantly, computational methods not only facilitate the separation of overlapping biosensor signals but also reduce phototoxicity by extracting biologically meaningful information from low-intensity images (Gómez-de-Mariscal et al., 2024). In addition, hyper-spectrum imaging and unmixing algorithms have increasingly been applied to the

clinical domain as reviewed by Rehman and Qureshi for various cancer types (Rehman & Qureshi, 2021).

While software innovations attract increasing attention, hardware progress remains indispensable for successful multiplex imaging. Advances in spectral detectors, tunable filters, and snapshot imaging systems directly improve unmixing performance (Li et al., 2013). This highlights the synergy between computational sophistication and hardware innovation to expand the capacity of multiplex live imaging.

Together, computational approaches expand the achievable multiplexing live imaging capacity by disentangling complex fluorescence emission spectra through methods ranging from classical linear unmixing to AI/ML-based unmixing. The development of user-friendly and robust computational tools for biological image analyses will further accelerate the biological and clinical applications of multiplex imaging.

Application of Multiplex Imaging in Cancer Research

Multiplex live imaging is highly valuable in many research fields where more than one cellular signaling pathway is dynamically intertwined to regulate downstream cellular behaviors. To highlight the advantages of multiplex imaging, we introduce representative applications in cancer research.

Cancer is characterized by the abundance and variation of defective signaling pathways. Their signaling activities exhibit intricate feedback and crosstalk regulations to drive oncogenesis and tumor progression (Fu et al., 2022; Lemmon & Schlessinger, 2010). Furthermore, cancer cells are highly variable among cells and across patients (tumor heterogeneity) in the tissue microenvironment, which gives rise to cancer cell clones of different phenotypes such as uncontrolled growth, invasion, metastasis and drug resistance (Hanahan & Weinberg, 2011). Given the multitude of deregulated signaling pathways in cancer, multiplex live imaging is pivotal in the research field.

Mitogen-activated protein kinase (MAPK) and PI3K-AKT pathways, two central signaling cascades in cancer, are often co-visualized using multiplex imaging because of their synergistic or compensatory roles in regulating cell proliferation, survival, and drug resistance (Mendoza et al., 2011). Maryu et al. showed cell cycle-dependent coordination of ERK and AKT activities in HeLa cells by spectral multiplexing of KTRs for ERK and AKT with a S/G2/M cell-cycle reporter, mCherry-hGem (Maryu et al., 2016). Likewise, Miura et al. multiplexed KTRs for JNK and p38 and showed cross-

inhibition of JNK by p38 leads to tumor cell heterogeneity (Miura et al., 2018). Chavez-Abiega et al. also used the KTRs in HeLa cells to reveal distinctive temporal ERK and AKT activity patterns triggered by G-protein-coupled receptor (GPCR) ligands (Chavez-Abiega et al., 2022).

Though the number of targets visualized by spectral multiplexing is generally limited to up to three targets, some studies show higher multiplicity by spatial and temporal multiplexing strategies, which is valuable in dissecting the heterogeneous features of cancer. Kaufmann et al. visualized 12 key cancer-related signaling pathways in A375 melanoma cells by spatial multiplexing (Kaufman et al., 2022). Yang et al. introduced a biosensor barcoding strategy in which both of the intracellular and intercellular multiplexing serve to assign specific biosensors to individual cells in a mixed population. They demonstrated the expandability of their strategy to multiple cancer cell lines including MCF7, U87MG, SiHa, and U2OS (J.-M. Yang et al., 2021). Ko et al. employed temporal multiplexing strategy using a click chemistry-based technology in living mice to remove fluorescence signals tagged to antibodies for sequential observations of multiple targets. In their method called scission-accelerated fluorophore exchange (SAFE), Ko et al. observed 12 immunological markers under intravital imaging of transplanted MC38 colon adenocarcinoma cells (Ko et al., 2022). Representing a multiplexing strategy not reliant on fluorescence, Chen et al. demonstrated a 14-plexed Raman spectroscopy targeting cell surface proteins such as HER2, endocytosis activity, and metabolic dynamics leveraging a devised Raman probe panel and a home-built whole-cell confocal Raman micro-spectroscopy (C. Chen et al., 2021).

Although studies applying multiplex live imaging to more than two signaling targets remain relatively limited, partly due to the technical challenges posed by tumor heterogeneity, integrating live imaging with ‘omics analyses is essential to overcome the inherent limitation of ‘omics approaches (Alieva et al., 2023). Ongoing advances in biosensor engineering, multiplexing strategies, and computational unmixing are expected to further expand the feasibility and performance of multiplex live imaging. Such progress will ultimately introduce a critical temporal dimension to the current framework of single-cell cancer biology.

Future Perspectives

Multiplex live imaging represents a powerful platform for interrogating complex cellular processes. Deciphering intricate signaling networks characterized by compensatory activity, feedback loops, and crosstalk requires simultaneous observation of multiple pathways, which is instrumental for understanding both physiological and pathological contexts. The pathology of cancer is profoundly influenced by heterogeneous genetic and post-transcriptional alterations, underscoring the indispensable role of multiplex live imaging in elucidating dynamic disease mechanisms.

Future progress in this field will likely be driven by several key directions: (1) the development of brighter and more sensitive fluorescent biosensors, especially those operating in the far-red and near-infrared spectra; (2) the integration of multiple photophysical and biochemical properties of fluorescent proteins beyond conventional intensity-based readouts; and (3) the retrospective reconstruction of signaling histories using genome editing-based recording systems (W. Chen et al., 2024).

Despite the ingenious strategies developed to maximize multiplexing capacity, none of the current multiplexing strategies, whether spatial, temporal, intercellular, or computational, are without trade-offs, as each entails inherent advantages and limitations. With existing methods, the number of simultaneously observable targets is generally limited to approximately ten distinct signals. Therefore, future innovations of multiplex methodologies are still expected to achieve a substantially higher level of multiplex imaging.

Ultimately, advancement of multiplex live imaging technologies will provide novel insights into biology and medicine by complementing static ‘omics approaches with dynamic temporal information, thereby bridging the gap between molecular networks and cellular behaviors in real time.

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Ethics approval and consent to participate

Ethics approval is not required in this study.

Patient consent for publication

Patient consent is not required in this study.

Figure legends

Figure 1. Multiplex live imaging strategies.

Summary of three approaches to achieve live imaging of multiple targets (multiplex live imaging) to overcome the limitation of available fluorescent spectral range. In spectral multiplexing, different fluorescence biosensors are expressed in identical cells. In spatial multiplexing, fluorescence biosensors are expressed in different subcellular locations of identical cells (intracellular multiplexing), or in different cells (intercellular multiplexing). In temporal multiplexing, multiple fluorescent probes are temporally switched for sequential image acquisitions or temporal features such as fluorescence lifetime are used as readouts.

Figure 2. Spectral multiplexing.

(A) The wavelength spectrum commonly used for live imaging (300 – 750 nm). Ultraviolet (< 400 nm) and far-red (> 700 nm) fluorescent proteins can expand the limitation of spectral multiplexing. As a representative ultraviolet and far-red fluorescent protein, the excitation (Ex) and emission (Em) spectrum of Sirius and iRFP670 are shown. The detection spectrum of conventionally used fluorescent proteins (EBFP, CFP, EGFP, YFP, and mCherry) are shown below. **(B)** Emission spectrum of the four fluorescent proteins (CFP, YFP, mKOκ, and mKate2) used for dual-FRET live imaging by Watabe et al. (Watabe et al., 2020). The detection wavelength ranges are shown below.

Figure 3. Intracellular multiplexing.

(A) Schematic showing different subcellular locations used for spatial multiplexing strategy. The subcellular locations and the commonly used tags are listed on the right. **(B)** Schematic of Signal Reporter Island (SiRIs) as an example of spatial multiplexing strategy. Linghu et al. live imaged the subcellular clusters of signal reporters that are subsequently subjected to immunostaining for the identification of the reporter types. Figure adapted from (Linghu et al., 2020).

Figure 4. Intercellular multiplexing.

Schematic of an example of intercellular multiplexing showing that different biosensors are expressed in each well of the multi-well plate on which the cells of the same origin are plated. The number of biosensors used for intercellular multiplexing is only limited by the number of wells in the multi-well plate.

Figure 5. Temporal multiplexing.

(A) Schematic of an example of temporal multiplexing strategy by reversibly switchable fluorescent proteins (rsFPs). Willig et al. combined EGFP and rsEGFP. Following the initial 488 nm laser to switch off rsEGFP, EGFP signal was detected.

After switching on rsEGFP, the sum signal of EGFP and rsEGFP was detected. The signal of rsEGFP was obtained by subtracting EGFP signal from the sum signal (Willig et al., 2021). **(B)** Unmixing of multiple reversibly switchable fluorescent protein signals by differential temporal decay patterns. Figure adapted from (Qian et al., 2023).

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Figure 1.

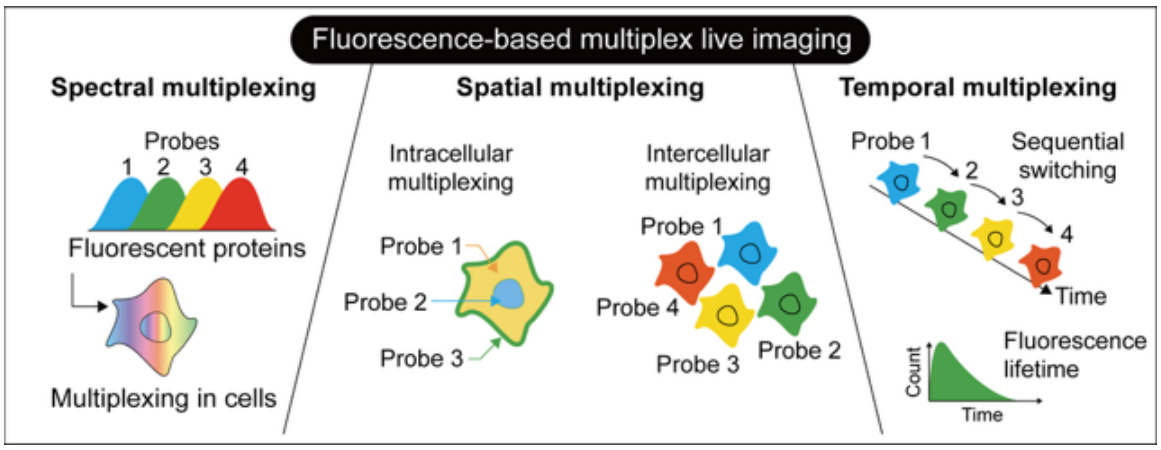

Fluorescence-based multiplex live imaging
Spectral multiplexing
Spatial multiplexing
Temporal multiplexing
Probes
1 2 3 4
Fluorescent proteins
Multiplexing in cells
Intracellular multiplexing
Probe 1
Probe 2
Probe 3
Intercellular multiplexing
Probe 1
Probe 4
Probe 3
Probe 2
Probe 1
2
3
4
Time
Sequential switching
Count
Time
Fluorescence lifetime

Figure 2.

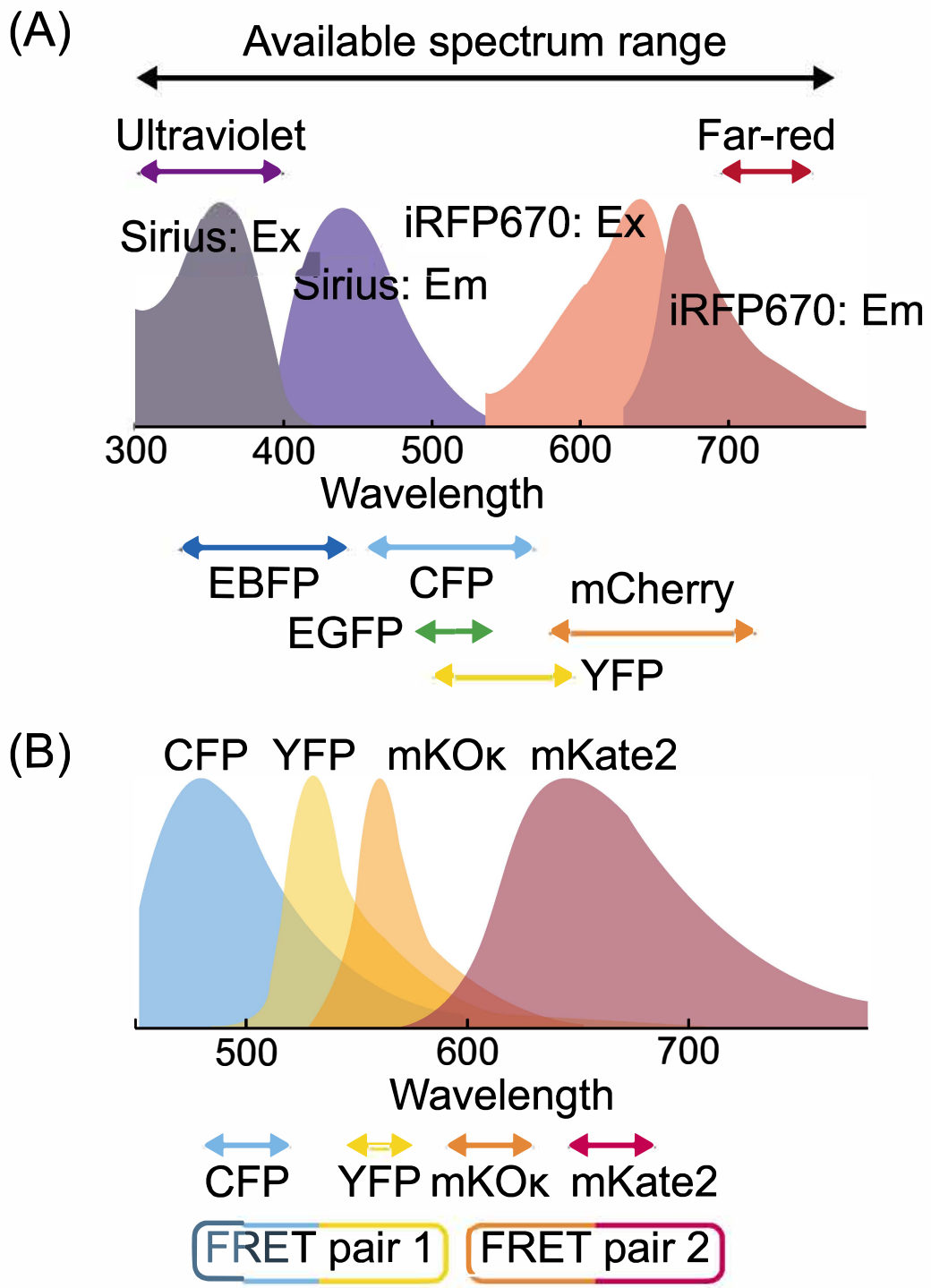

(A)
Available spectrum range
Ultraviolet
Far-red
Sirius: Ex
Sirius: Em
iRFP670: Ex
iRFP670: Em
300
400
500
600
700
Wavelength
EBFP
CFP
mCherry
EGFP
YFP
(B)
CFP YFP mKOκ mKate2
500
600
700
Wavelength
CFP
YFP
mKOκ
mKate2
FRET pair 1
FRET pair 2

Figure 3.

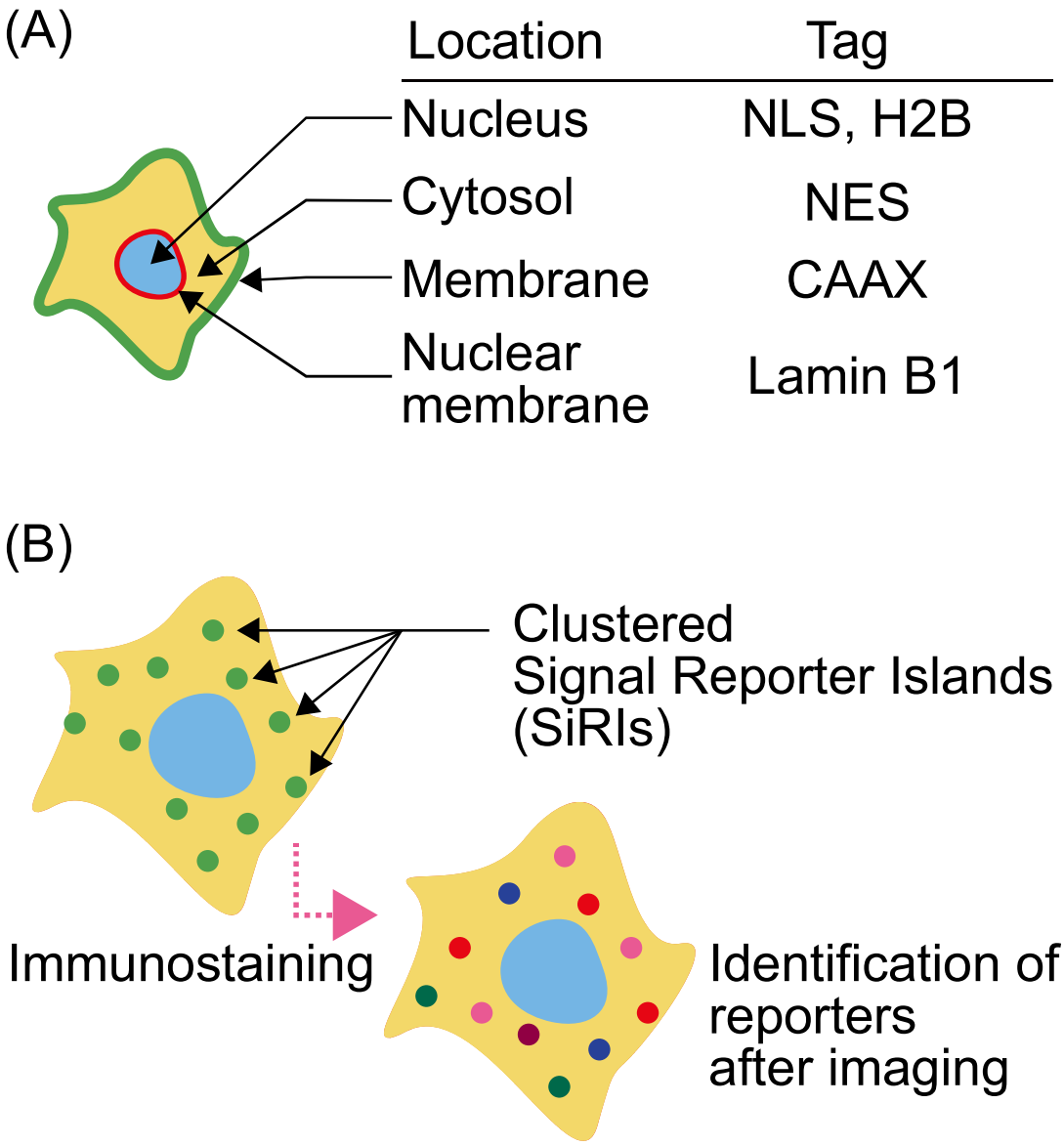

(A)
Location
Tag
Nucleus
NLS, H2B
Cytosol
NES
Membrane
CAAX
Nuclear membrane
Lamin B1
(B)
Clustered Signal Reporter Islands (SiRIs)
Immunostaining
Identification of reporters after imaging

Figure 4.

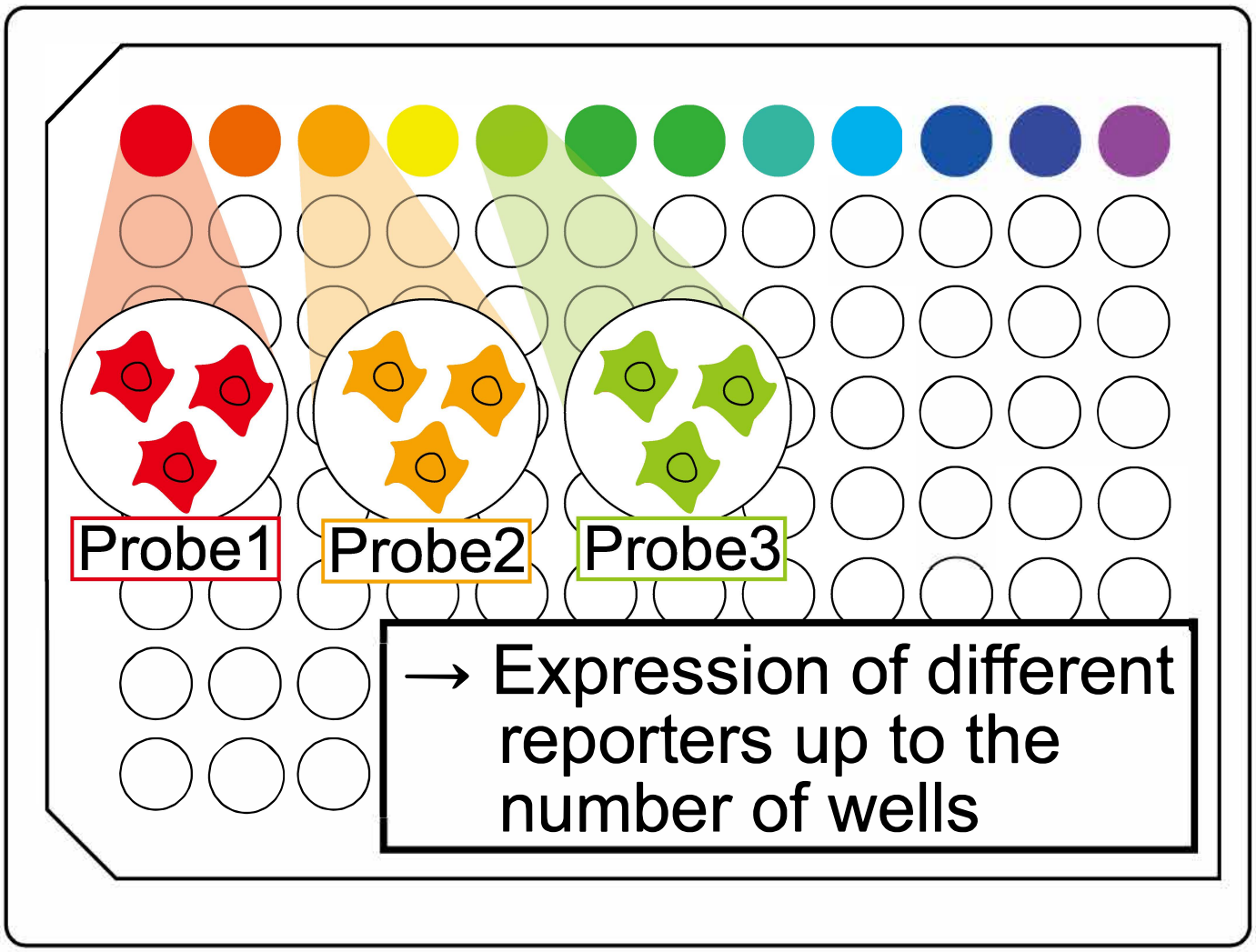
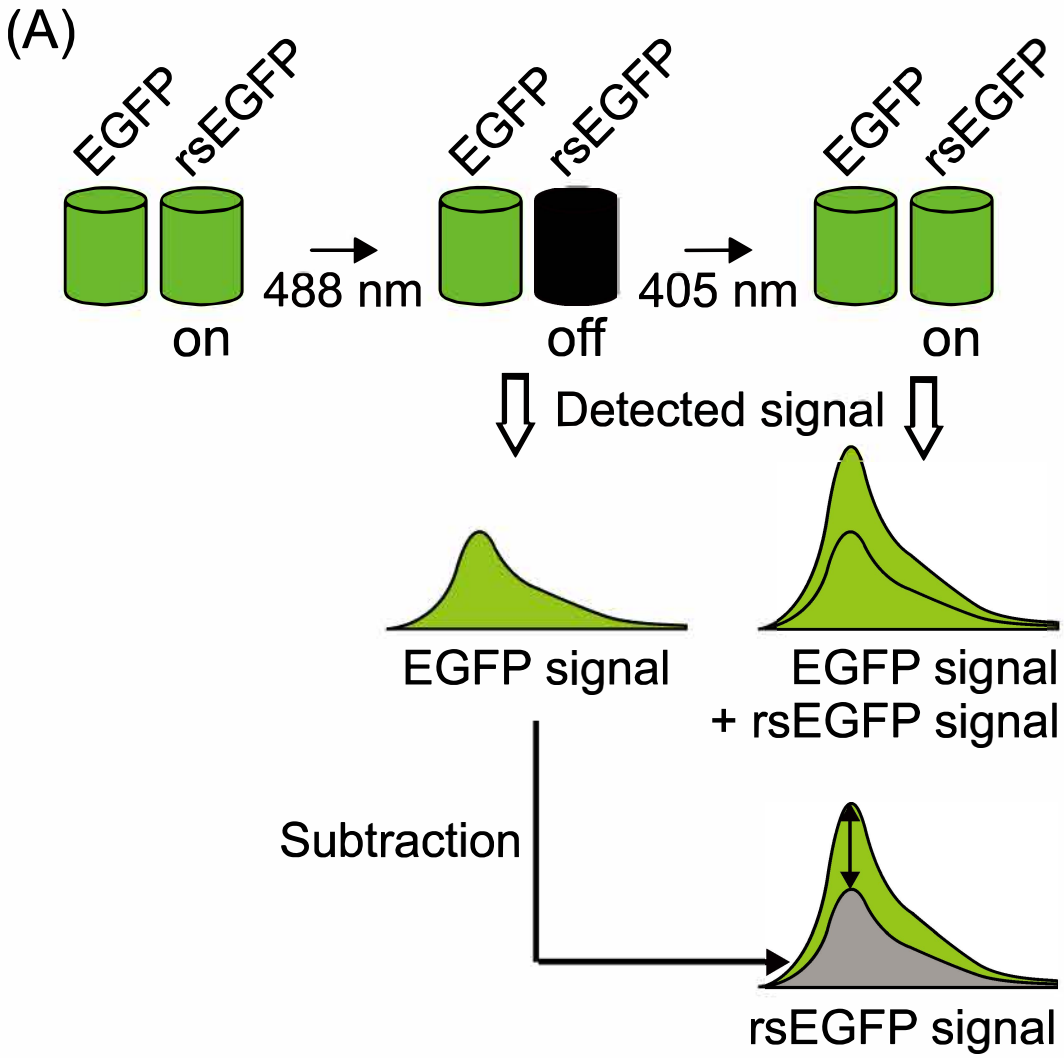
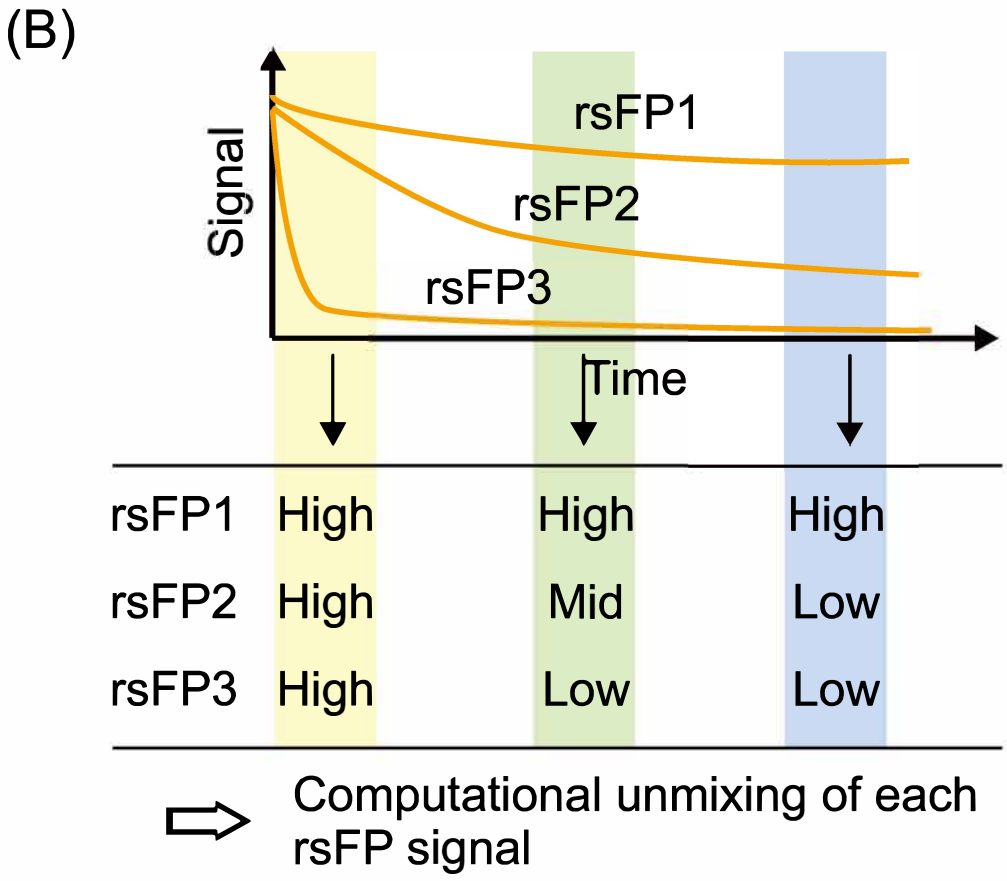

Probe1
Probe2
Probe3
→ Expression of different reporters up to the number of wells

Figure 5.


(A)
EGFP
rsEGFP
on
488 nm
off
405 nm
on
Detected signal
EGFP signal
EGFP signal
+ rsEGFP signal
Subtraction
rsEGFP signal


(B)
Signal
rsFP1
rsFP2
rsFP3
Time
rsFP1 High High High
rsFP2 High Mid Low
rsFP3 High Low Low
Computational unmixing of each
rsFP signal