A Critical and Comparative Review of Fluorescent Tools for Live-Cell Imaging

Elizabeth A. Specht,[1,2] Esther Braselmann,[1,2] and Amy E. Palmer[1,2]

[1]Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80303; email: amy.palmer@colorado.edu

[2]BioFrontiers Institute, University of Colorado, Boulder, Colorado 80303

Annu. Rev. Physiol. 2017. 79:93–117

First published online as a Review in Advance on November 16, 2016

The *Annual Review of Physiology* is online at physiol.annualreviews.org

This article's doi: 10.1146/annurev-physiol-022516-034055

Keywords

fluorescent proteins, fluorescent dyes, probes, live-cell imaging, cellular dynamics

Abstract

Fluorescent tools have revolutionized our ability to probe biological dynamics, particularly at the cellular level. Fluorescent sensors have been developed on several platforms, utilizing either small-molecule dyes or fluorescent proteins, to monitor proteins, RNA, DNA, small molecules, and even cellular properties, such as pH and membrane potential. We briefly summarize the impressive history of tool development for these various applications and then discuss the most recent noteworthy developments in more detail. Particular emphasis is placed on tools suitable for single-cell analysis and especially live-cell imaging applications. Finally, we discuss prominent areas of need in future fluorescent tool development—specifically, advancing our capability to analyze and integrate the plethora of high-content data generated by fluorescence imaging.

1. INTRODUCTION

Fluorescent tools have launched biological research into a new realm of understanding of cellular processes and dynamics at the single-cell level. These tools are enabling the characterization of stochasticity and heterogeneity exhibited by biological systems, which could not adequately be probed by techniques that rely on bulk analysis of populations of cells. Early applications of fluorescent tools entailed monitoring protein dynamics, which continues to be a field of active development. Recent advances in photoactivatable and photoswitchable probes enable more sophisticated measurements of protein stability and turnover, and multiplexing capabilities are pushing the field toward high-throughput proteomics using data-rich fluorescent techniques. The past decade has experienced a surge in new tools for nucleic acid imaging, and single-molecule detection used in combination with highly multiplexed labeling is enabling unprecedented quantitative global analysis of cellular responses.

Fluorescent sensors are also increasingly providing insight into what could previously be called the "dark matter" of the cellular milieu: small molecules, secondary metabolites, metals, and ions. Never before had these essential molecular species been visualized on the single-cell level. Tools can now probe global shifts in cellular state that are not well captured by any single molecular indicator, such as membrane potential, cellular division, and tissue differentiation.

This review provides a broad overview of well-established fluorescent tools, with an eye toward recent developments and emerging technologies, and it refers the reader to more comprehensive and detailed reviews on individual techniques and applications. We begin with a discussion of general classes of fluorophores and their advantages and disadvantages for various applications. We then discuss methods for labeling a molecule of interest with a fluorescent moiety—including fluorescent protein fusions, incorporation of fluorescent moieties through nonnatural amino acid substitution, chemical labeling, and antibody labeling—emphasizing applications in live cells. We briefly review applications for monitoring proteins, which are already well established, and then focus on the extension of these techniques to high-throughput proteomics and screening. Progress in visualizing nucleic acids is discussed, followed by an overview of recent developments in fluorescent sensors for small molecules and cell state. Finally, we discuss areas of need for future development, emphasizing the need for improved automated image analysis and multiparametric systems biology methods to handle the exponential growth of data.

2. NEW ADVANCES IN THE DEVELOPMENT OF FLUOROPHORES

The potential to peer inside cells to visualize, track, and quantify molecules, ions, proteins, nucleic acids, and biochemical reactions has led to the development of a vast collection of small molecular fluorophores and fluorescent proteins. Choice of the specific fluorophore or even class of fluorophore (small molecule versus fluorescent protein) depends on several factors, including the nature of the experiment, potential for perturbation of the molecule or cellular state of interest, and optical properties of the fluorophore. Fluorescent dyes are small and exhibit favorable optical properties, such as brightness, photostability, and narrow bandwidth relative to fluorescent proteins. They can be designed to be membrane permeable to illuminate intracellular milieu or membrane impermeable to report on extracellular species, although controlling localization is not necessarily trivial.

Fluorescent dyes can be chemically modified through organic synthesis. Thus, they can be tailored to the cellular environment or illumination method. There are now hundreds of commercially available organic dyes that span the visible wavelength range (1, 2). Many of these are built from a smaller number of core scaffolds; innovative and combinatorial synthetic routes to

generate and functionalize the core scaffold can lead to a huge expansion in the available tools (3, 4). Advances in rational design strategies have recently expanded dye scaffolds, permitting new chemistry and extending the spectral range. Inspired by insights from molecular modeling, a simple structural modification recently yielded increases in both brightness and photostability (with a nearly twofold improvement in quantum efficiency), with generalizability across the spectrum of live-cell imaging fluorophore candidates (5).

A noteworthy recent addition to the suite of small-molecule fluorophores is the near-infrared (IR) silicon-rhodamine dyes (6) and their extension to DNA stains (7) and cytoskeletal stains (8) with improved spectral properties for long-term and in vivo imaging. Another novel class of fluorogenic molecules consists of the carbofluoresceins and carborhodamines (9), which can be caged or masked to modulate their fluorescent properties. This unique attribute makes these molecules valuable as sensors or reporters, as well as for superresolution microscopy applications.

The two most frequently cited limitations of organic fluorophores for live-cell imaging are the lack of molecular specificity—necessitating sophisticated approaches for attaching fluorophores to biomolecules—and the challenge of ensuring cell permeability (10). The use of fluorescent proteins (FPs) as fluorophores circumvents both these limitations due to their genetic encodability. Since the 1990s, FPs have been subjected to extensive protein engineering to tune their optical properties (wavelength range, brightness, and photostability), as well as biochemical properties (rate of protein folding and chromophore maturation, pKa, oligomerization state, robustness in fusions and in oxidizing environments). The engineering and properties of traditional FPs were extensively reviewed (11, 12). Notable recent additions to the FP toolbox include mNeonGreen (13), mRuby3 (14), FusionRed (15), mGarnet (16), and oxFPs (17). A recent publication (18) provides a useful quantitative comparison of the optical properties and cellular performance of over 40 FPs.

3. METHODS OF TAGGING MOLECULES OF INTEREST

3.1. Fluorescent Protein Fusions

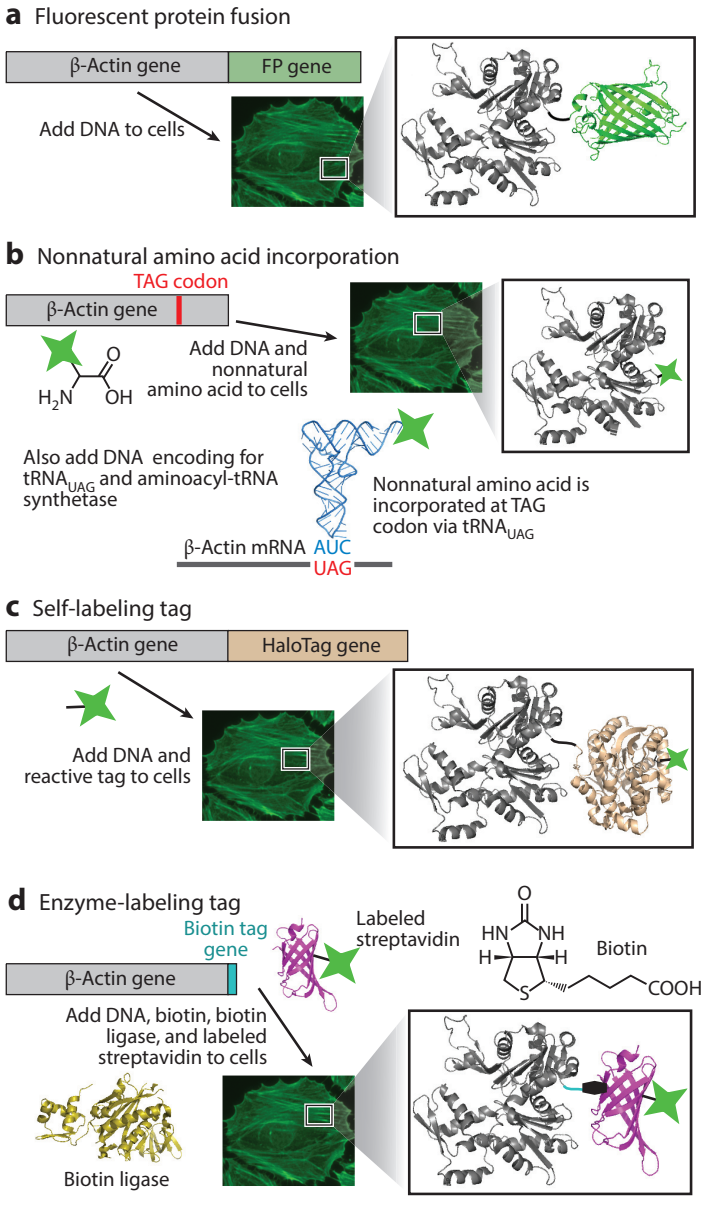
The simplicity and ease of creating FP fusions have revolutionized our ability to peer into cells and visualize dynamic processes in real time (**Figure 1**). However, perturbation of the stability, localization, or function of the tagged protein of interest remains a perennial concern, especially for proteins that assemble into larger complexes. Cross-reference between FP-tagged proteins and immunofluorescence can be used to validate whether the fusion proteins behave similarly to the endogenous proteins. One study systematically evaluated the localization of over 500 FP-protein fusions and found, perhaps surprisingly, that localization of 80% of the FP-tagged proteins agreed with immunofluorescence data (19). The antibodies used in this study were generated and validated by the Human Protein Atlas Project. Despite the strong overlap, there were notable discrepancies, including one quarter of proteins whose localization was different between the N-terminal and the C-terminal FP fusion. These cases often corresponded to proteins with multiple isoforms, or proteins that are targeted to the mitochondria or secretory pathway, in which an N-terminal fusion disrupted the signal peptide.

Other studies have noted instances where fusions are tolerated at neither terminus but are stable internally within a flexible region of the protein of interest (20). It is important to note that localization is not predictive of a function, and further assessment of native protein function must also be pursued. Linker choice can also significantly affect the stability and disruptiveness of an FP fusion; strategies to optimize linkers within protein fusions were recently reviewed (21).

Fluorescent proteins also exhibit oligomerization or aggregation, despite years of engineering toward soluble monomeric forms. Aggregation concerns are especially pertinent when targeted to

organelles (22), or when the FP fusion is highly expressed, as is often the case when transfected as an exogenous construct using high copy number plasmids and strong promoters. New strategies to tag endogenous loci through genome editing [via TALENs, zinc finger nucleases, or most recently CRISPR/Cas (23, 24)] can alleviate aggregation from overexpression, while also ensuring that the FP fusion more closely mimics physiologically relevant expression levels and patterns.

For proteins that do not tolerate bulky fusions with a full FP, an alternative strategy is to utilize a split-FP system wherein a single beta strand is fused to the protein of interest (25). The remainder of the FP is expressed separately and combines with the fused strand to reconstitute



the intact fluorescent protein. This strategy is even more amenable to CRISPR/Cas targeting to endogenous loci, as the small split-FP tag can be encoded on a single oligonucleotide template for high-efficiency gene tagging (26).

3.2. Fluorescent Nonnatural Amino Acids

Incorporating fluorophores directly into proteins via nonnatural amino acids enables labeling proteins in live cells. This strategy addresses some of the concerns of functional perturbation from bulky FP fusions. Several fluorescent amino acids, including L-(7-hydroxycoumarin-4-yl)-ethylglycine (27), 2-amino-3-[5-(dimethylamino)naphthalene-1-sulfonamide]-propanoic acid (28), and 6-propionyl-2-(*N*,*N*-dimethylamino)napthalene (29), were incorporated at amber codons by evolving orthogonal aminoacyl-tRNA synthetases to specifically recognize the fluorescent amino acid. This approach has also been used to introduce a new fluorophore into cyan fluorescent protein, altering the spectral properties of the FP by inducing fluorescence resonance energy transfer (FRET) between the biological and chemical fluorophores (30). Alternatively, amino acids with chemical handles can be incorporated into the protein, followed by chemoselective reactions to attach a fluorophore. These methods were recently comprehensively reviewed (31).

Side-by-side comparisons have elucidated cases where FP fusions impaired the function of the protein of interest, whereas nonnatural amino acid substitutions were well tolerated (32). However, incorporation efficiency of nonnatural amino acids is often context-dependent and affected by neighboring primary sequence. One alternative is to incorporate the fluorescent nonnatural amino acid in the form of a small N- or C-terminal tag that was already optimized for high-efficiency incorporation, yet is less obtrusive than an FP fusion (33).

3.3. In Situ Chemical Labeling

There are two main approaches for direct chemical attachment of a fluorophore to a protein: self-labeling tags and enzymatic peptide labeling. Self-labeling tags tend to be larger—similar in size to FPs—so they may perturb the target. Enzymatic peptide labeling requires only a small 13–15 amino acid peptide to be introduced into the protein of interest, and the kinetics of labeling tend to be rapid, but such labeling increases the risk of off-target labeling. Furthermore, it requires the introduction of an additional element, bringing the labeling system to three components: the probe, the dye, and the enzyme. With either labeling scheme, the dye should be nonfluorescent

Figure 1

Methods of fluorescently tagging proteins. As an example, tagging of β-actin by various tools is presented where the genetically encoded parts are drawn as bars (to scale). (*a*) FPs can be fused at the N- or C-termini of a protein of interest. The structures of β-actin (PDB ID 2BTF) and GFP (PDB ID 1EMA) are shown at the same relative scale. (*b*) A modified tRNA (loaded by an evolved aminoacyl-tRNA synthetase) can incorporate an unnatural amino acid at the amber (TAG) codon. A representative tRNA structure (ID 1EHZ) is shown to illustrate the incorporation of an unnatural amino acid in response to the amber codon (UAG) in the mRNA. (*c*) The HaloTag can be genetically fused to a protein of interest (HaloTag, PDB ID 4KAA). The HaloTag consists of a modified dehalogenase that covalently binds to a membrane-permeant synthetic ligand. (*d*) A short biotin tag sequence is genetically fused to a protein of interest. Biotin is then bound to the tag by biotin ligase, which in turn tightly binds labeled streptavidin. The structures of β-actin and streptavidin (PDB ID 4JNJ) in the fusion construct are shown at the same relative scale (biotin ligase: PDB ID 1BIA). Abbreviations: FP, fluorescent protein; GFP, green fluorescent protein.

outside of the context of the protein, or it should be highly permeable such that excess dye can be washed out of cells after labeling to reduce background fluorescence. These methods were recently covered in great detail in several excellent reviews (34, 35).

3.3.1. Self-labeling tags. The most commonly employed self-labeling tags are SNAP-tag (36), CLIP-tag (37), HaloTag (38), and TMP-tag (39). All are enzymes that can covalently couple a chemical moiety to itself if provided the appropriate substrate. These reactions are orthogonal to one another and occur relatively quickly compared to enzymatically labeled peptide tags (see Section 3.3.2). A wide variety of chemically functionalized substrates have been developed for each platform, including a plethora of fluorophores with diverse spectral properties (see 31 for a comprehensive review). Strategies to reduce the background of unbound probe include self-quenching probes, whereby the quencher is removed when the fluorophore is chemically coupled to the protein of interest (40).

Another class of self-labeling tags consists of tetra-cysteine and tetra-serine tags, which bind dyes noncovalently. Tetra-cysteine tags bind the cell-permeable biarsenical dyes FlAsH and ReAsH (41). In theory these dyes exhibit a high signal-to-noise ratio because the unbound dyes are not fluorescent (42). However, in practice, nonspecific association of the dye with cellular proteins limits the signal-to-noise ratio. Tetra-serine tags, which bind the bis-boronic dye RhoBo, result in less toxicity but increased off-target labeling, as some endogenous human proteins contain the SSPGSS tag consensus sequence (43).

3.3.2. Enzymatic labeling tags. Biotin ligases of bacterial origin recognize a 15-residue biotinylation site that is distinct from mammalian biotin ligases; thus, adding the bacterial biotinylation tag and introducing the ligase into mammalian cells allow specific biotinylation of a protein of interest (44). Strepavidin-fluorophore conjugates can then detect this moiety but are subject to background detection of endogenously biotinylated proteins. Alternatively, biotin ligases from other species including yeast are able to accept alkyne and azide derivatives of biotin, directly facilitating click chemistry fluorophore attachment (45), although the presence of endogenous biotin may decrease labeling efficiency. Click chemistry refers to a series of biocompatible reactions that enable a biomolecule to be conjugated to a reporter molecule. The most common click chemistry reactions involve copper(I)-catalyzed cycloaddition between an azide and an alkyne, strain-promoted [also known as (3+2)] cycloaddition, and thiol-ene reactions. Copper-free click chemistry, which approximates the kinetics and efficiency of the canonical copper-catalyzed reaction, should be used for live-cell applications to reduce toxicity (46). Lipoic acid ligase from *Escherichia coli* has been engineered to attach fluorescent moieties directly to the protein of interest (47, 48), but can only tolerate small fluorophore substrates that excite and emit at short, high-energy wavelengths that are typically prohibitively damaging for live-cell or long-term imaging (34).

3.4. Fluorescent Antibody Labeling

Due to their large size (~150 kDa), antibodies have historically been limited to applications in fixed, permeabilized cells. However, the specificity of antibodies is useful for monitoring endogenous proteins in cells, so several recent strategies have been developed for antibody-based live-cell imaging. These approaches typically utilize only a portion of a full-length antibody, either the antigen binding fragment (F_{ab}, ~55 kDa), the single-chain variable fragment (scFV, ~28 kDa), or the smallest recognition unit called a nanobody or V_HH domain (~12 kDa), derived from single-chain camelid antibodies (49). Because antibodies can show off-target binding, they should be verified in cells in which the protein of interest is knocked out.

3.4.1. Fluorobodies, chromobodies, and FabLEM. The most common uses of antibodies for live-cell imaging entail genetic fusions between an antibody fragment and an FP. Fusions between an scFV, comprising the V_L and V_H domains, and an FP are typically called fluorobodies (50). The field of histone modification labeling has termed this design a Mintbody (modification-specific intracellular antibody) when used to detect a specific posttranslational or epigenetic modification (51). Chromobodies entail a similar design, whereby the scFV is replaced with a smaller nanobody. These antibody derivatives bind less strongly to their target than does a full-length antibody, enabling better dynamics for live-cell imaging (51). Exchange with a cellular probe pool may mitigate photobleaching, although this can decrease fluorescence contrast due to elevated background.

The advantages of antibody-FP fusions relative to direct FP fusions are monitoring of endogenous proteins and the ability to detect specific posttranslational modifications. Antibody-FP fusions may also be advantageous in cases where proper folding of the target protein is impeded by FP fusion. Because antibodies recognize endogenous untagged proteins, the target protein will be translated and folded prior to recognition by the antibody-FP fusion. However, the possibility still exists that antibody binding can trigger conformational change or alter the function of the target protein (50, 52).

FabLEM (F_{ab} live endogenous modification) uses monovalent antigen binding fragment (F_{ab}) domains obtained by digestion from monoclonal antibodies, which are then conjugated to a small-molecule fluorescent dye. The conjugated F_{ab} can be introduced into cells either by microinjection or by bead loading, a technique using 75–500 μm glass beads to create small, transient tears in the cell membrane (53). With either method, cells can be imaged immediately. Background fluorescence may be high if the concentration of F_{ab} conjugates is not optimized, but it can be minimized using temporal averaging in the imaging process (54, 55). The advantage of this approach is the brightness of small-molecule fluorophores; however, imaging can only occur for 1–2 days before the conjugates are diluted out through cell division. A systematic study empirically determined the most suitable dyes for single- or multichannel FabLEM imaging (56).

3.4.2. Engineered scFVs as fluorogen activating proteins. Directed evolution of scFVs via yeast or phage display can be used to generate fluorogen activating proteins (FAPs). As the name suggests, FAPs are proteins that bind a nonfluorescent dye and cause an increase in fluorescence emission, typically due to stabilization and rigidification of the dye molecule. These are selected with specificity to individual fluorogenic dyes including malachite green and thiazole orange (57). Some promiscuous variants of scFV FAPs are capable of binding any member of a broad set of cyanine dyes, thus eliciting fluorescence at many possible wavelengths spanning the visible and near-IR spectra (58). This feature confers experimental flexibility, as the emission wavelength can be altered by changing the dye rather than reengineering the scFV.

Recently, engineered scFV FAPs were used to generate far-red probes for STED superresolution microscopy with spectral parameters and quantum yields on par with ATTO dyes (59). Even greater signal-to-noise ratios can be achieved via multidonor amplification in fluorogenic dendrons, comprising several Cy3 donors coupled to a single scFV-activated malachite green fluorogen (60). It remains to be seen whether the FAP approach is feasible with smaller antibody fragments such as nanobodies.

3.4.3. Antibody scaffolding. The high specificity and binding affinity of antibodies (and scFV fragments) to their respective epitopes were recently exploited to generate FP scaffolds that can achieve single-molecule resolution. The SunTag was the first demonstration of this approach, where a linear epitope tag of up to 24 copies is fused to a protein of interest and an

scFV-GFP (green fluorescent protein) fusion is expressed simultaneously within the cell (61). The tag then recruits multiple GFPs to the protein of interest, providing signal amplification via scaffolding. SunTag24X fused to a membrane-targeting domain resulted in puncta 18-fold brighter than membrane-targeted GFP alone, indicating that the majority of the epitopes are populated by scFV-GFP (61). This tag is less bulky than direct fusion with multiple FPs, but the unstructured nature of the tags and their repetitive sequences pose problems for expression and stability (62), limiting their use to variants with fewer epitope copies.

To address the low stability of this design, tandem epitope tags were incorporated into multiple locations on the hyperstable GFP variant, superfolder GFP, which improved the stability and expression of the tag (62). This design, "spaghetti monster FP" or smFP, was used with bead loading of antibody-dye conjugates to achieve significantly increased brightness for single-molecule tracking. Two orthogonal versions—based on mRuby2 and mWasabi, which do not cross-react with anti-GFP antibodies—were also developed (62).

4. MONITORING PROTEIN DYNAMICS

Proteins have arguably been more extensively studied using fluorescent tools than has any other cellular constituent, in part because proteins are amenable to tagging by all of the methods outlined in Section 3. FP fusions are widely used to study protein localization on a global scale. Nearly all open reading frames in yeast (63) and several hundred in human cells were systematically evaluated in this way, often with multiple fusions (for example, one each at the N-terminus and C-terminus) to assess perturbations in trafficking or stability (64). Developments in photocontrollable FPs and photoactivatable dyes have facilitated superresolution microscopy of exquisitely detailed cellular structures using protein tagging (65, 66). We highlight a handful of popular techniques from the long history of protein study with fluorescent tools and provide references for more detailed reviews. We then discuss developments at the intersection of protein imaging tools with high-throughput screening and analysis for fluorescent proteomic analysis.

4.1. Protein Turnover

In the past 10–15 years, several techniques have been developed to study protein dynamics, not just abundance and distribution within cells. Two broad classes of fluorescent timers were developed: photoswitchable (photoactivatable or photoconvertible) FPs suitable for pulse-chase experiments and shorter time frames (minutes) and timers that rely on maturation kinetics of FPs for longer time frames (hours) (67). Monomeric fluorescent protein timers (FTs) rely on a time-dependent blue-to-red conversion within the chromophore, which can be used to determine rates of protein synthesis and turnover. Structural studies have shed light on the mechanism of this conversion, identifying critical residues that can be manipulated to engineer new FTs with more diverse time frames (68).

Tandem fluorescent protein timers (tFTs) exploit variable chromophore maturation kinetics among different FPs, typically using a rapidly maturing GFP and a slower-maturing red fluorescent protein (69). Recently, evidence suggests that the GFP component of tFTs is not efficiently degraded within the cell, leaving tFT fragments that may preclude the use of the timers to determine protein turnover rates (70). GFP variants that are less resistant to proteasomal degradation have been suggested for future tFT development.

4.2. High-Throughput Microscopy for Proteomic Analysis

Historically, proteomics studies have relied heavily on mass spectrometry, providing insight into protein abundance and posttranslational modification frequency. With advanced fluorescence

imaging tools, we now can approach proteomics with single-cell resolution, temporal information, and additional parameters such as cell cycle stage (see Section 6.3.2), shape, and size. The two main challenges for this application are developing high-throughput and automated methods to quantitate and integrate high-content microscopy data (see Section 7) and creating comprehensive FP-tagged strain libraries. Progress on both of these fronts is reviewed in 71.

Beginning in the mid-2000s, researchers began using GFP-tagged libraries to screen strains harboring fluorescent versions of nearly every known protein in the yeast genome (63). Global proteomic shifts in response to media changes (72), chemical perturbations, genetic mutations (73), and DNA damaging agents (74) were probed on millions of cells. The localization and abundance of thousands of proteins can be quantified using automated image analysis, and flux diagrams can be used to represent the proteomic flux induced by the experimental condition (73). Data can be gathered using automated microscopy in multiwell dishes, by flow cytometry, or by imaging flow cytometry (75) incorporating fluorescence barcoding (76) to differentiate pooled members of an FP-tagged library. Comparisons between proteomic data gathered by microscopy, flow cytometry, and more traditional proteomics methods were recently reviewed (77).

Thus far, the majority of fluorescent global proteomics work was performed in yeast due to the lack of comprehensive tagged libraries in other cells, but the CRISPR-Cas tagging strategies discussed in Section 3.1 suggest that these studies will rapidly be expanded to mammalian cells. Subsets of the human proteome were examined with high-throughput microscopy; for example, 53 nuclear proteins were assessed for abundance, localization, mobility, and protein–protein interactions using fluorescence correlation spectroscopy (78). The ability to simultaneously monitor and account for other morphological parameters (such as cell shape, size, contact with neighboring cells, and mitotic state) greatly improves the reproducibility of conclusions drawn from large-scale screens (79).

5. MONITORING NUCLEIC ACID DYNAMICS

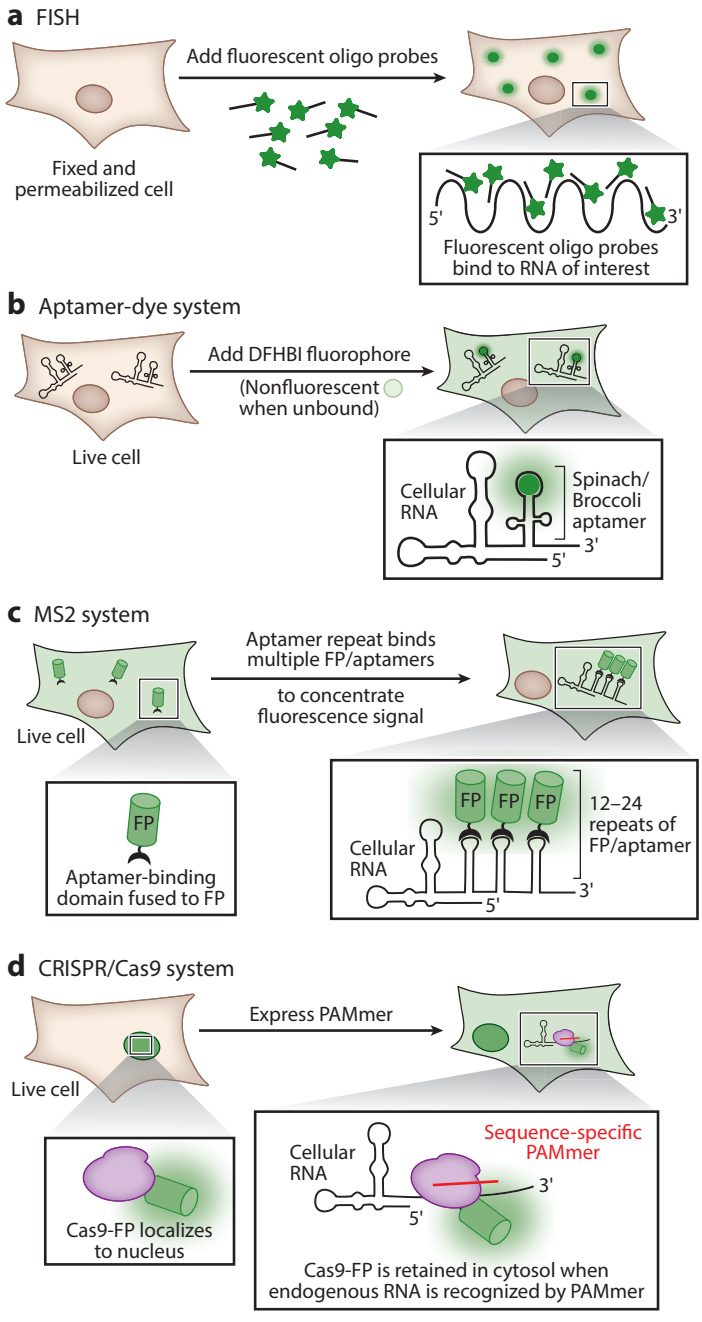
Methods to assess dynamic cellular responses have historically been heavily focused on measurements of protein dynamics due to the relative abundance of protein detection tools. However, several fluorescent tools were developed in recent years to probe DNA and RNA abundance and localization in fixed cells, as well as to monitor their dynamics in living cells. Furthermore, high-resolution techniques now permit monitoring and mechanistic insight into DNA replication, transcription (80), translation (81), and DNA repair (82), which were all recently reviewed. Used in combination, these approaches facilitate a systems-level understanding of cellular response and regulation.

5.1. Approaches for Monitoring RNA

RNA, including noncoding RNA species, is implicated in a vast number of dynamic regulatory processes, and it is no longer viewed as merely a messenger for protein production. In this section, we focus on fluorescent tools that enable spatial resolution of RNA molecules within cells, which have advanced significantly within the last several years (**Figure 2**).

5.1.1. Fixed-cell techniques for RNA detection. RNA-FISH (fluorescence in situ hybridization), which was developed in the 1980s, is the method of choice for RNA detection in fixed cells. Recent developments in single-molecule detection and multiplexing capacity have dramatically increased the amount of information obtainable by this method. The first demonstration of single-molecule FISH (smFISH) in 1998 relied on long, multiply labeled probes that proved difficult to synthesize (83), limiting widespread adoption. Smaller, singly labeled probes were developed in 2008 (84).

More recently, powerful strategies to multiplex smFISH have emerged. A simple technique is to photobleach the sample in between multiple hybridizations, allowing multiple RNA species to be visualized within a single sample or cell (85). A more elegant strategy is to use error-robust combinatorial labeling, in effect assigning a barcode to each RNA of interest. This technique allows highly multiplexed smFISH, with over 1,000 RNA species reliably detected with 14 rounds



of hybridization (86). These approaches harbor significant promise for developing complex systems biology models to predict global cellular responses and for characterizing the nature of cellular heterogeneity.

5.1.2. Live-cell RNA probes. There are several categories of live-cell RNA imaging probes, most of which provide spatial resolution within the cell. Molecular beacons, dye aptamers, and nanoflares all rely on small-molecule fluorescent dyes, often in combination with a quencher to reduce signal in the absence of RNA target recognition. Molecular beacons and dye aptamers exhibit fluorescence at the site of the RNA target, whereas nanoflares release the fluorescent moiety upon target recognition. Other approaches utilize fluorescent proteins fused to DNA-binding proteins, either aptamer-binding proteins such as MS2, PP7, or pumilio1, or very recently the RNA-guided binding protein Cas9 of CRISPR-Cas (87). The aptamer strategies require engineering the target RNA of interest, whereas molecular beacons, nanoflares, and rCas9 (ssRNA-recognizing Cas9) can recognize native target sequences.

To generate a molecular beacon, a FRET pair or a fluorophore-quencher pair is attached to the ends of a hairpin oligonucleotide, where the probe sequence complementary to the target resides in the hairpin loop. Upon hybridization with the target, the fluorophore is spatially separated from the quencher. Despite their simplicity, molecular beacons are of limited utility in live-cell imaging due to the challenge of loading them into cells and their susceptibility to degradation (88).

Nanoflares employ a similar concept except that the fluorophore is tethered to a gold nanoparticle, which acts as a quencher (89). The RNA target exhibits a higher binding affinity for the probe than the fluorophore-containing oligonucleotide, thus displacing it and spatially separating the fluorophore from the quencher. In this case, the target RNAs are sequestered at the gold nanoparticle, which may perturb functionality. Additionally, the fluorophore is released, so target RNA abundance can be quantified but intracellular localization cannot be tracked.

Several groups have characterized RNA aptamers that activate fluorogenic dyes (90, 91), but these have not been used extensively in live cells due to high background signal and cellular toxicity (92). The Spinach system was designed to overcome some of these limitations; this platform utilizes the small-molecule fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), which interacts with a small RNA aptamer evolved through SELEX (systematic evolution of ligands by exponential enrichment). Subsequent generations were developed, including Broccoli (93) and iSpinach (94), which were evolved and selected within cells for improved signal and robustness in vivo. The structure, mechanism, and characteristics of these RNA aptamers were recently reviewed (95).

Several naturally evolved RNA-binding proteins are used to target fluorescent proteins to RNAs of interest. Two orthogonal systems are commonly used, adapted from bacteriophage proteins that bind specific RNA stem-loops: MCP (MS2 coat protein) and PCP (PP7 coat protein) (reviewed

Figure 2

Methods of fluorescently labeling RNA in fixed or live samples. (*a*) Endogenous RNA is labeled by sequence-specific fluorescent oligo probes that bind the RNA of interest in fixed permeabilized cells. (*b*) A small aptamer (called Spinach/Broccoli) is genetically incorporated as a fusion of the RNA of interest, and fluorescence activation is achieved after binding of the cell-permeable small molecule DFHBI. (*c*) Short aptamers are incorporated at the 3′ end of the RNA of interest in series. Each aptamer binds an aptamer-binding protein fused to one or several FPs. (*d*) An FP is fused to a genetically modified version of Cas9 that remains nuclear, unless it is retained in the cytosol upon binding to an endogenous RNA of interest via a sequence-specific PAMmer. Abbreviation: FP, fluorescent protein; PAMmer, protospacer adjacent motif-presenting oligomer.

in 96). Typically, several copies of the aptamer are introduced to a single transcript to increase fluorescence contrast, resulting in a bulky system that may interfere with normal RNA function. Recent work in yeast suggests that the stem-loop aptamers cause aberrant degradation of the target RNAs (97). Background is also high from unbound GFP-MCP/PCP fusions, although this can be reduced by using a split-FP system and adjacent, orthogonal aptamers (98). The pumilio1 RNA-binding protein is also used for this purpose, using unique pumilio1 domains that recognize specific aptamer sequences (99).

Finally, a CRISPR/Cas RNA imaging platform was recently described, combining the native sequence-targeting abilities of base-pairing probes with the malleability of engineered protein sensors. Although Cas9 nuclease is typically used to bind DNA, it can also recognize ssRNA if provided a complementary DNA oligonucleotide at the intended target site (100). This strategy was extended to RNA imaging in live cells using a GFP-Cas9 fusion and was found to cause minimal disruption to RNA levels or localization as verified by RNA-FISH (87).

5.2. Approaches for Monitoring DNA

Although DNA is not as dynamic in abundance or cellular localization as RNA, its configuration and accessibility are important indicators of activity. Furthermore, DNA replication and repair are critical checkpoints for cell cycle progression, and thus tools for monitoring these processes are useful for biomedical applications such as developing diagnostics for DNA damage and cancer.

5.2.1. Tracking genomic loci. The abundance (e.g., copy number) and location/accessibility of genomic loci are important indicators of cellular state and may signal aberrant activity. Similar to RNA, single-molecule FISH can be used on fixed cells to quantify and spatially resolve genomic loci, but it cannot be used in live cells. Recently, CRISPR/Cas was also adapted for live-cell genomic imaging using a Cas9-GFP fusion (101). Labeling of repetitive telomeric regions indicates that the probe can find its target within highly compacted chromatin. However, there are concerns about background fluorescence and interference with target gene transcription (101). The ability to track specific loci in real time during cell division may enable more sophisticated characterization of phenomena such as meiotic recombination.

5.2.2. Monitoring DNA damage and repair. Fluorescent probes marking sites of DNA damage provide important insight into the mechanisms by which cells sense and respond to genomic injury (82). Common examples include FP-tagged end-binding proteins that congregate at sites of double-stranded DNA breaks. Comet-FISH is another common technique for analyzing DNA damage, combining the Comet (single-cell gel electrophoresis) method (102) with FISH to determine whether DNA damage has occurred near a genomic locus of interest (reviewed in 103). Fluorescent probes were also developed to detect particular mechanisms of DNA repair, including base excision repair (104) and nucleotide excision repair (105), which may have applications in drug-resistance monitoring during chemotherapy (104) or to detect predisposition to cancer due to faulty DNA repair machinery (105). High-throughput fluorescent microscopy has also expanded our knowledge of DNA repair pathways, even in well-studied organisms; a yeast GFP-fusion library was screened in response to chemical mutagens and revealed two novel DNA damage responses (106).

6. MONITORING BIOCHEMICAL CHANGES AND CELLULAR STATE

Fluorescent sensors, probes, and indicators are molecules that report on the dynamics of analytes, enzymatic activities, or cellular states through a change in fluorescence properties. Such tools have

employed a wide range of mechanisms by which cellular dynamics are converted into a fluorescence readout, including FRET, intensity changes, translocation between subcellular compartments, spectral shifts, and small-molecule–responsive transcription factors that drive expression of a fluorescent reporter. Probes can also be reaction-based, wherein the analyte of interest mediates a chemical reaction as a catalyst or selective reagent (107). Given the growing evidence that cells encode information in the temporal activities of their signaling molecules, tracking the dynamic biochemistry of living cells is more popular than ever (108). The huge diversity of cellular sensors cannot be reviewed in detail here. Below, we highlight the main types of sensors and refer readers to recent reviews for further detail (**Table 1**). We also chronicle the development of a few sensor platforms in more detail to highlight successful approaches that may serve as roadmaps for fluorescent tool optimization in other areas.

6.1. Small-Molecule, Metabolite, and Ion Sensors

Analyte sensors represent the largest class of sensors. Small-molecule, protein, transcriptional reporter, and more recently RNA-based sensors were developed for analytes as diverse as cations (Ca^{2+}, Zn^{2+}, and other alkali, alkaline earth, and transition metals), neurotransmitters, metabolites, and second messengers. The *Molecular Probes Handbook* (1) provides a useful reference that catalogues the strengths and limitations of commercially available small-molecule probes. We refer readers to a thorough review of genetically encoded biosensors from 2011 (109), and transcriptional reporters from 2015 (110). Metal ion sensors were also recently comprehensively reviewed (111). RNA riboswitches have inspired a new class of small-molecule and metabolite sensors that rely on fluorescence modulation upon interaction with an RNA aptamer, often based on the design of the Spinach aptamer described under RNA imaging tools above (112).

The oldest and most mature family of analyte sensors encompasses small-molecule and genetically encoded calcium indicators (GECIs). Ca^{2+} is a ubiquitous second messenger that serves as a key node in many signaling pathways. Its intricate spatial distribution and exquisitely regulated dynamics have long lured researchers into developing ever more sophisticated tools for monitoring the spatial and temporal patterns of Ca^{2+} signals from living cells to whole organisms. Since the introduction of synthetic Ca^{2+} indicators in the 1980s and genetically encoded indicators in the 1990s, probes have been subjected to intense iterative optimization. Although synthetic dyes remained the gold standard for many years with respect to dynamic range, response kinetics, and indicator linearity, GECIs have recently come of age and surpass synthetic dyes for many applications (113). However, a recent comparison of small-molecule Ca^{2+} indicators and GCamP6 sensors revealed the superiority of small-molecule dyes, particularly Cal-520 and Rhod-4, for monitoring local Ca^{2+} signals such as Ca^{2+} puffs induced by IP3-mediated release of Ca^{2+} from the endoplasmic reticulum (114).

The two most common classes of GECIs are the single FP-based indicators (GCamP platform), which are intensity-based and rely on collection of a single fluorescence channel, and FRET-based indicators, which are ratiometric and require dual-channel recording. Both platforms were subjected to extensive iterative optimization to improve performance metrics (113, 115, 116). A major breakthrough in GECI performance came from insights derived from the analysis of crystal structures (117, 118) and large-scale mutagenesis and screening strategies (119–121). Screening and functional assessment upon electrical stimulation in neurons was particularly valuable for identifying sensors with improved response kinetics and amplitudes (119). The best in class GECIs are now sufficiently sensitive to permit in vivo imaging in a wide range of model systems, including worm, zebrafish, fly, mouse, and nonhuman primates (113, 115).

Table 1 Select examples of tools to monitor cellular dynamics by fluorescence microscopy to illustrate design principles

Monitored item	Name/description of tool	Type of detection	Genetic encoding	Key advantages/disadvantages
Ca^{2+}	GCamPs/GECIs (113)	Intensity change (single FP sensor) or FRET-based	Yes	Plus: permits long-term measurement of Ca^{2+} transients in transgenic organisms Minus: may perturb endogenous calcium dynamics (114)
	Small-molecule Ca^{2+} indicators	Intensity change or spectral shift in dye	No	Plus: fast response time (114) Minus: intracellular concentration of dye can be very high
Kinase activity	KTRs (126)	Change in localization (nucleus versus cytoplasm) of single FP reporter	Yes	Plus: multiplexing for detection of up to 4 different kinase activities possible
Voltage change across a membrane	Genetically encoded voltage sensors (based on conformational or photophysical change of sensing domain) (133, 134)	Intensity change (single FP sensor) or FRET-based	Yes	Plus: targeting to small pool of neurons in live animals possible Minus: slow response times compared to small-molecule probes
	Small-molecule-based voltage sensors (130)	Intensity change or FRET-based	No	Minus: delivery to membrane is difficult; partitioning in other membranes likely (130)
Cell-cycle stages	Fucci (fluorescent, ubiquitination-based cell-cycle indicator) (137)	Cell-cycle state-dependent degradation of FP-reporters (green/red color change at M–G1 transition, yellow at G1–S transition)	Yes	Minus: requires delivery of two reporters
	CDK2-based localization change (138)	Change in localization (nucleus versus cytoplasm) of single FP reporter	Yes	Plus: single color allowing it to be multiplexed with other probes
$NADH/NAD^+$	SoNar (144)	Conformational change of single FP fused to $NADH/NAD^+$-sensing domain	Yes	Plus: insensitive to changes in pH; ratiometric
Molecular crowding	GimRET (145)	FRET-based	Yes	Plus: can be targeted to different organelles to monitor crowding

Abbreviations: FP, fluorescent protein; FRET, fluorescence resonance energy transfer; GECI, genetically encoded calcium indicator; GimRET, crowding-sensitive FRET pair; KTR, kinase translocation reporter.

Although state-of-the-art green GCamPs permit robust measurement of Ca^{2+} transients, most notably for monitoring neuronal activity, there are some limitations to in vivo use. In particular, poor tissue penetration of blue excitation light and spectral overlap with optogenetic tools for controlling neuronal signals have led to concerted efforts to expand the color palette of single FP sensors into the red. Whereas early generation red calcium indicators suffered from diminished performance compared to their green counterparts, sensitive indicators based on R-CamP (derived from mRuby) and R-GECO (derived from mApple) were recently developed (122).

6.2. Sensors for Enzymatic Activities

A large number of fluorescent sensors of enzymatic activities were developed, with the aim of characterizing complex cellular signaling dynamics and regulatory pathways at the single-cell level. G-protein coupled receptors (GPCRs) play a fundamental role in cell signaling, and fluorescent tools were developed to study GPCR activation (123) as well as to understand interactions between GPCRs and their ligands (124). Mechanisms and examples of kinase activity sensors were recently reviewed, as well as their extension to high-throughput screening for drug discovery (125). Recently, a generalizable method for producing specific kinase activity sensors was developed in which a self-phosphorylating kinase is fused to an FP, with phosphorylation sites flanked by a nuclear export signal and a nuclear localization signal (126). These kinase translocation reporters reside in the nucleus when inactive and are exported to the cytosol upon activation. Small-molecule fluorescent probes were also engineered to detect protease and glycosidase activity (127). Protease activity probes, many of which are fluorescent and suitable for live-cell imaging, were recently extensively reviewed (128), as were a suite of other fluorescent protein-based tools for assessing posttranslational modifications (129).

6.3. Probes to Assess Cell State

Cells often respond to environmental stimuli with a global change in their cellular state, which may not be fully captured by monitoring individual proteins, transcripts, enzymatic activities, or small-molecule concentrations. Fluorescent probes exist for a handful of these states, including indicators of membrane potential, cell cycle, redox state, and probes for molecular crowding within cellular compartments.

6.3.1. Voltage and membrane potential sensors. Although the voltage clamp is the gold standard for defining ion currents and measuring voltage to study how single neurons process information, the optical detection of voltage enables detection of neuronal activity in tissue (130). For decades, the only available tools were voltage- and/or Ca^{2+}-sensitive dyes (VSDs, reviewed in 131) and more recently the genetically encoded Ca^{2+} indicators (132). The past half-decade has witnessed the rapid development of a suite of genetically encoded voltage indicators, based on voltage-sensitive phosphatases (VSP) or rhodopsins as the voltage-sensing domain. Fusions between a VSP and an FP, usually circularly permuted, generate intensiometric sensors whereby voltage-induced conformational change alters the intensity of FP fluorescence. Alternatively, FRET-based designs allow ratiometric measurements. VSP-based designs generally suffer from slow response kinetics and narrow dynamic range, but mutagenesis and rational design continue to reveal sensor variants with improved properties (133).

Rhodopsin exhibits natural fluorescence and is used as an intensiometric sensor on its own; however, the brightness is increased when an FP is fused to serve as a FRET donor, generating

a so-called FRET-opsin (134). Steric hindrance between the FP and the membrane-embedded rhodopsin makes optimization of the FRET distance difficult, but slight alterations in the linker length and composition can drastically improve the speed and magnitude of the response (135). Two excellent recent reviews summarize the response kinetics, dynamic range, sensitivity, brightness, and other experimentally relevant parameters of all the currently characterized voltage probes (133, 134). Voltage sensors are often used in combination with optogenetic tools engineered from photoreceptors, typically phytochromes or cryptochromes that respond conformationally to light. Optogenetic tools are beyond the scope of this review but are comprehensively described elsewhere (136).

6.3.2. Cell-cycle progression. As measurements have moved from bulk analysis of a heterogeneous cellular population to the monitoring of individual cells, cell-cycle tracking has become highly desirable. The most commonly used cell-cycle sensor is Fucci, which exhibits red fluorescence in G_1 that shifts to green fluorescence in S, G_2, and M phases (137). Fucci is nuclear localized, facilitating automated analysis. A more recent cell-cycle sensor detects changes in CDK2 activity by monitoring nuclear to cytoplasmic translocation of a fluorescent reporter. This sensor is nuclear localized when CDK2 is low (G_0 and G_1), evenly distributed throughout the nucleus and cytoplasm in moderate CDK2 activity (S phase), and exclusively cytosolic when CDK2 activity is high (G_2 and M) (138). Automated analysis is performed by analyzing the ratio of nuclear fluorescence signal to the signal within a cytoplasmic ring immediately outside the nuclear boundary. This sensor is ideal for multiplexing with other probes, as it occupies a single fluorescence channel.

6.3.3. Redox and ROS probes. A number of probes were developed to report on the redox environment within the cell by monitoring redox couples, such as the ratio of reduced to oxidized glutathione (GSH:GSSG), the presence of reactive oxygen or nitrogen species (ROS, RNS), or key metabolites in redox metabolism such as NADH and NAD^+. Small-molecule fluorophore-based redox probes that detect a variety of ROS and RNS were recently reviewed (139, 140). These probes lack subcellular specificity and are not ratiometric; there are also concerns about their stability and reversibility. Genetically encoded sensors were developed for ROS, GSH:GSSG, and NADH/NAD^+. Several recent reviews highlight the history of sensor development and limitations of different platforms with respect to specificity, sensitivity, and pH-dependence (141–143). More recently a new probe (SoNar) was developed for robustly tracking NAD^+ and NADH based on insertion of circular permuted YFP into a Rex protein and subsequent sensor optimization. This probe is bright, pH-resistant, and fast, enabling high-throughput metabolic screening of compounds that alter energy metabolism (144).

6.3.4. Molecular crowding. Recently, the first fluorescent indicator of molecular crowding was developed, exploiting the observed differential sensitivity of various FPs to protein concentration in vitro (145). A hypersensitive point-mutant of YFP was paired with CFP, resulting in a ratiometric reporter in which crowding alters FRET between the two FPs. This observation should serve as a reminder that a plethora of cellular parameters may affect the responsiveness or dynamic range of fluorescent sensors, and proper controls and calibrations are essential for interpreting sensor readouts.

7. AREAS OF NEED FOR FUTURE DEVELOPMENT

With rapid advances in fluorescent tool development and improvements in microscopy platforms, the most significant challenges currently facing the field are new methods for processing and

interpreting the vast amounts of data being generated by fluorescence microscopy. Below we discuss two notable areas of need: strategies to incorporate complex data into systems-level modeling and user-friendly software for automated image analysis.

7.1. Integrative Systems Biology Approaches for Cellular Dynamics

"Omics" studies have historically relied on methods that separate the biomolecules of interest from a bulk population of cells, such as mass spectrometry, microarrays, next-generation DNA sequencing, or RNA seq. Although these methods are extremely powerful, cells have evolved complex and redundant mechanisms for regulating cellular functions in response to stress and other stimuli. Moreover, there is growing recognition of both heterogeneity (146) and dynamics (108) in cellular states and cellular signaling pathways that call for single-cell, time-lapse measurements to elucidate mechanisms of regulation. Simultaneous interrogation of multiple levels of cellular dynamics (e.g., RNA level, protein level, and metabolite level) may reveal novel regulatory mechanisms that govern cellular dynamics. In this respect, one limitation of fluorescence microscopy is the small number of parameters that can be imaged simultaneously due to spectral overlap of optical probes. A recent approach for circumventing this limitation is the development of a phenotypic screen to identify the ideal reporter cell lines whose phenotypic profile captures the effect of different classes of drugs (147). Alternatively, a common approach involves tracking dynamics of biomarkers of interest using appropriate probes, followed by fixing and staining for a broader repertoire of biomarkers that help define features of the cellular state, albeit at a fixed point in time (138). Using immunofluorescence, cells can be reprobed with a variety of different markers to build a high-dimensional data set.

As described throughout this review, tools have now been developed to monitor a vast array of cellular constituents in live cells using fluorescence microscopy. Now, a true systems-level biological understanding of cellular dynamics is conceivable by measuring these constituents in parallel, with temporal and intracellular resolution within single cells (148). What is needed, then, is a multiplexed approach that integrates information from several monitoring techniques into a systems-level model of cellular dynamics (148). Thus far, studies incorporating simultaneous monitoring of multiple cellular constituents are fairly limited, but recent work is beginning to reveal some of the parameters contributing to what was previously considered to be biological noise.

In addition to fluorescence data, other morphometric parameters can be quantified from the images, such as cell size, morphology, and mitotic stage (149). In mammalian cells, the cellular microenvironment was shown to account for approximately 60–80% of cell-to-cell variability with regards to viral susceptibility (79). Accounting for these factors also increases reproducibility in RNAi screens (150). Once comprehensive experimental data sets were gathered, elemental principles were established for building and refining systems biology models that most accurately reflect—and ideally, predict—the measured experimental parameters (151). However, complex high-dimensional data sets—for example, measurements on thousands of proteins and thousands of transcripts—will require correspondingly complex model development strategies.

7.2. Automated Image Analysis and Multiparametric Data Analysis

Similar to many fields of biological research, fluorescence-based imaging is undergoing an infusion of new technologies that permit massively increased data generation. Automated time-lapse microscopy, which can generate temporally rich data sets for thousands of cells within a single experiment, and the advent of imaging flow cytometry (75) demand more advanced image analysis algorithms. The amount of data permits detection of more subtle or nuanced phenotypes, but in many cases the data generated exceed our current capabilities for quantitative analysis.

A typical analysis pipeline includes image preprocessing or filtering to reduce noise, segmentation and region of interest determination, and finally feature extraction. Time-lapse imaging also often includes particle or object tracking to monitor migratory cells or subcellular components over time; particle tracking algorithms were recently reviewed (152). A review of 15 free software tools for fluorescent image analysis evaluated their performance based on criteria such as documentation, data management, visualization, and flexibility, and placed each package on a usability/functionality graph (153). Each program exhibits strengths and weaknesses, and the best choice depends on the application at hand as well as the ability of the scientist to modify existing scripts, suggesting that life scientists should receive greater training in computational analysis and coding. Alternatively, the most effective way to process high-content imaging data sets may be in collaboration with computer scientists and engineers using custom-built computational tools (154).

Streamlining image analysis across many types of experiments is particularly difficult, as studies are likely to vary widely in the attributes studied, cell line/morphology used, and thresholds for defining cellular responses. Some approaches utilize automated algorithms in combination with oversight from a human observer to validate calls and correct errors, which has proven highly successful for segmentation, tracking, and lineage analysis (155). Another elegant approach is to employ supervised machine learning algorithms to allow the researcher to adapt a standardized analysis to their individual experimental conditions (156, 157). One set of annotated data is used to train the software, and the researcher can make corrections throughout the analysis to iteratively improve the accuracy of the program (158). The field would benefit greatly from an extensive collection of these programs from which researchers can select the analytical tools closest to their needs and train them on their own data.

There is also a strong need to make multiparametric data analysis and visualization methods more amenable to high-content imaging applications, such that multiple observational parameters can be analyzed for their independent and combined contributions to a phenotype of interest (159). Platforms for five-dimensional (5D) image analysis (3D imaging data with time and fluorescence channel as additional variables) have recently been developed for tracking neural stem cell lineage within intact tissue samples, providing information on interactions with vasculature in the tissue microenvironment (160). This field has progressed rapidly, but a significant need still exists for novel software packages and open-source malleable algorithms for high-throughput, automated quantitative image analysis.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors would like to acknowledge generous financial support from NIH grants GM084027 and DP1-GM114863, as well as the Human Frontiers Science Project.

LITERATURE CITED

1. Spence MTZ, Johnson ID. 2010. *Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies*. Carlsbad, CA: Life Tech. Corp. 11th ed.
2. Lavis LD, Raines RT. 2008. Bright ideas for chemical biology. *ACS Chem. Biol.* 3(3):142–55

3. Lavis LD, Raines RT. 2014. Bright building blocks for chemical biology. *ACS Chem. Biol.* 9(4):855–66
4. Wysocki LM, Lavis LD. 2011. Advances in the chemistry of small molecule fluorescent probes. *Curr. Opin. Chem. Biol.* 15(6):752–59
5. Grimm JB, English BP, Chen J, Slaughter JP, Zhang Z, et al. 2015. A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nat. Methods* 12(3):244–50
6. Lukinavičius G, Umezawa K, Olivier N, Honigmann A, Yang G, et al. 2013. A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat. Chem.* 5(2):132–39
7. Lukinavičius G, Blaukopf C, Pershagen E, Schena A, Reymond L, et al. 2015. SiR-Hoechst is a far-red DNA stain for live-cell nanoscopy. *Nat. Commun.* 6:8497
8. Lukinavičius G, Reymond L, D'Este E, Masharina A, Göttfert F, et al. 2014. Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat. Methods* 11(7):731–33
9. Grimm JB, Sung AJ, Legant WR, Hulamm P, Matlosz SM, et al. 2013. Carbofluoresceins and carborhodamines as scaffolds for high-contrast fluorogenic probes. *ACS Chem. Biol.* 8(6):1303–10
10. Giepmans BNG, Adams SR, Ellisman MH, Tsien RY. 2006. The fluorescent toolbox for assessing protein location and function. *Science* 312:217–24
11. Snapp EL. 2009. Fluorescent proteins: a cell biologist's user guide. *Trends Cell Biol.* 19(11):649–55
12. Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA. 2010. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* 90(3):1103–63
13. Shaner NC, Lambert GG, Chammas A, Ni Y, Cranfill PJ, et al. 2013. A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat. Methods* 10(5):407–9
14. Bajar BT, Wang ES, Lam AJ, Kim BB, Jacobs CL, et al. 2016. Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting. *Sci. Rep.* 6:20889
15. Shemiakina II, Ermakova GV, Cranfill PJ, Baird MA, Evans RA, et al. 2012. A monomeric red fluorescent protein with low cytotoxicity. *Nat. Commun.* 3:1204
16. Hense A, Prunsche B, Gao P, Ishitsuka Y, Nienhaus K, Nienhaus GU. 2015. Monomeric garnet, a far-red fluorescent protein for live-cell STED imaging. *Sci. Rep.* 5:18006
17. Costantini LM, Baloban M, Markwardt ML, Rizzo M, Guo F, et al. 2015. A palette of fluorescent proteins optimized for diverse cellular environments. *Nat. Commun.* 6:7670
18. Cranfill PJ, Sell BR, Baird MA, Allen JR, Lavagnino Z, et al. 2016. Quantitative assessment of fluorescent proteins. *Nat. Methods* 13:557–62
19. Stadler C, Rexhepaj E, Singan VR, Murphy RF, Pepperkok R, et al. 2013. Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. *Nat. Methods* 10(4):315–23
20. Giraldez T, Hughes TE, Sigworth FJ. 2005. Generation of functional fluorescent BK channels by random insertion of GFP variants. *J. Gen. Physiol.* 126(5):429–38
21. Chen X, Zaro JL, Shen W-C. 2013. Fusion protein linkers: property, design and functionality. *Adv. Drug Deliv. Rev.* 65(10):1357–69
22. Costantini LM, Snapp EL. 2013. Fluorescent proteins in cellular organelles: serious pitfalls and some solutions. *DNA Cell Biol.* 32(11):622–27
23. Ratz M, Testa I, Hell SW, Jakobs S. 2015. CRISPR/Cas9-mediated endogenous protein tagging for RESOLFT super-resolution microscopy of living human cells. *Sci. Rep.* 5:9592
24. Stewart-Ornstein J, Lahav G. 2016. Dynamics of CDKN1A in single cells defined by an endogenous fluorescent tagging toolkit. *Cell Rep.* 14(7):1800–11
25. Cabantous S, Terwilliger TC, Waldo GS. 2005. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat. Biotechnol.* 23(1):102–7
26. Kamiyama D, Sekine S, Barsi-Rhyne B, Hu J, Chen B, et al. 2016. Versatile protein tagging in cells with split fluorescent protein. *Nat. Commun.* 7:11046
27. Wang J, Xie J, Schultz PG. 2006. A genetically encoded fluorescent amino acid. *J. Am. Chem. Soc.* 128(27):8738–39
28. Summerer D, Chen S, Wu N, Deiters A, Chin JW, Schultz PG. 2006. A genetically encoded fluorescent amino acid. *PNAS* 103(26):9785–89

29. Lee HS, Guo J, Lemke EA, Dimla RD, Schultz PG. 2009. Genetic incorporation of a small, environmentally sensitive, fluorescent probe into proteins in *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* 131(36):12921–23
30. Kuhn SM, Rubini M, Müller MA, Skerra A. 2011. Biosynthesis of a fluorescent protein with extreme pseudo-Stokes shift by introducing a genetically encoded non-natural amino acid outside the fluorophore. *J. Am. Chem. Soc.* 133(11):3708–11
31. Lang K, Chin JW. 2014. Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins. *Chem. Rev.* 114(9):4764–806
32. Charbon G, Brustad E, Scott KA, Wang J, Løbner-Olesen A, et al. 2011. Subcellular protein localization by using a genetically encoded fluorescent amino acid. *ChemBioChem* 12(12):1818–21
33. Abe R, Shiraga K, Ebisu S, Takagi H, Hohsaka T. 2010. Incorporation of fluorescent non-natural amino acids into N-terminal tag of proteins in cell-free translation and its dependence on position and neighboring codons. *J. Biosci. Bioeng.* 110(1):32–38
34. Dean KM, Palmer AE. 2014. Advances in fluorescence labeling strategies for dynamic cellular imaging. *Nat. Chem. Biol.* 10(7):512–23
35. Cserép GB, Herner A, Kele P. 2015. Bioorthogonal fluorescent labels: a review on combined forces. *Methods Appl. Fluoresc.* 3(4):042001
36. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. 2003. A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol.* 21(1):86–89
37. Gautier A, Juillerat A, Heinis C, Corrêa IR, Kindermann M, et al. 2008. An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol.* 15(2):128–36
38. Los G V, Encell LP, McDougall MG, Hartzell DD, Karassina N, et al. 2008. Halotag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3(6):373–82
39. Miller LW, Cai Y, Sheetz MP, Cornish VW. 2005. In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag. *Nat. Methods* 2(4):255–57
40. Sun X, Zhang A, Baker B, Sun L, Howard A, et al. 2011. Development of snap-tag fluorogenic probes for wash-free fluorescence imaging. *ChemBioChem* 12(14):2217–26
41. Adams SR, Tsien RY. 2008. Preparation of the membrane-permeant biarsenicals FlAsH-EDT_2 and ReAsH-EDT_2 for fluorescent labeling of tetracysteine-tagged proteins. *Nat. Protoc.* 3(9):1527–34
42. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, et al. 2002. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J. Am. Chem. Soc.* 124(21):6063–76
43. Halo TL, Appelbaum J, Hobert EM, Balkin DM, Schepartz A. 2009. Selective recognition of protein tetraserine motifs with a cell-permeable, pro-fluorescent bis-boronic acid. *J. Am. Chem. Soc.* 131(2):438–39
44. Howarth M, Takao K, Hayashi Y, Ting AY. 2005. Targeting quantum dots to surface proteins in living cells with biotin ligase. *PNAS* 102(21):7583–88
45. Slavoff SA, Chen I, Choi Y-A, Ting AY. 2008. Expanding the substrate tolerance of biotin ligase through exploration of enzymes from diverse species. *J. Am. Chem. Soc.* 130(4):1160–62
46. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, et al. 2007. Copper-free click chemistry for dynamic in vivo imaging. *PNAS* 104(43):16793–97
47. Uttamapinant C, White KA, Baruah H, Thompson S, Fernández-Suárez M, et al. 2010. A fluorophore ligase for site-specific protein labeling inside living cells. *PNAS* 107(24):10914–19
48. Cohen JD, Thompson S, Ting AY. 2011. Structure-guided engineering of a Pacific Blue fluorophore ligase for specific protein imaging in living cells. *Biochemistry* 50(38):8221–25
49. De Meyer T, Muyldermans S, Depicker A. 2014. Nanobody-based products as research and diagnostic tools. *Trends Biotechnol.* 32(5):263–70
50. Kaiser PD, Maier J, Traenkle B, Emele F, Rothbauer U. 2014. Recent progress in generating intracellular functional antibody fragments to target and trace cellular components in living cells. *Biochim. Biophys. Acta* 1844(11):1933–42
51. Kimura H, Hayashi-Takanaka Y, Stasevich TJ, Sato Y. 2015. Visualizing posttranslational and epigenetic modifications of endogenous proteins in vivo. *Histochem. Cell Biol.* 144(2):101–9

52. Gueorguieva D, Li S, Walsh N, Mukerji A, Tanha J, Pandey S. 2006. Identification of single-domain, Bax-specific intrabodies that confer resistance to mammalian cells against oxidative-stress-induced apoptosis. *FASEB J.* 20(14):2636–38
53. McNeil PL, Warder E. 1987. Glass beads load macromolecules into living cells. *J. Cell Sci.* 88(Pt. 5):669–78
54. Hayashi-Takanaka Y, Yamagata K, Wakayama T, Stasevich TJ, Kainuma T, et al. 2011. Tracking epigenetic histone modifications in single cells using Fab-based live endogenous modification labeling. *Nucleic Acids Res.* 39(15):6475–88
55. Sato Y, Mukai M, Ueda J, Muraki M, Stasevich TJ, et al. 2013. Genetically encoded system to track histone modification in vivo. *Sci. Rep.* 3:2436
56. Hayashi-Takanaka Y, Stasevich TJ, Kurumizaka H, Nozaki N, Kimura H. 2014. Evaluation of chemical fluorescent dyes as a protein conjugation partner for live cell imaging. *PLOS ONE* 9(9):e106271
57. Szent-Gyorgyi C, Schmidt BF, Schmidt BA, Creeger Y, Fisher GW, et al. 2008. Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nat. Biotechnol.* 26(2):235–40
58. Ozhalici-Unal H, Pow CL, Marks SA, Jesper LD, Silva GL, et al. 2008. A rainbow of fluoromodules: a promiscuous scFv protein binds to and activates a diverse set of fluorogenic cyanine dyes. *J. Am. Chem. Soc.* 130(38):12620–21
59. Yates BP, Peck MA, Berget PB. 2013. Directed evolution of a fluorogen-activating single chain antibody for function and enhanced brightness in the cytoplasm. *Mol. Biotechnol.* 54(3):829–41
60. Szent-Gyorgyi C, Schmidt BF, Fitzpatrick JAJ, Bruchez MP. 2010. Fluorogenic dendrons with multiple donor chromophores as bright genetically targeted and activated probes. *J. Am. Chem. Soc.* 132(32):11103–9
61. Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD. 2014. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* 159(3):635–46
62. Viswanathan S, Williams ME, Bloss EB, Stasevich TJ, Speer CM, et al. 2015. High-performance probes for light and electron microscopy. *Nat. Methods* 12(6):568–76
63. Huh W-K, Falvo JV, Gerke LC, Carroll AS, Howson RW, et al. 2003. Global analysis of protein localization in budding yeast. *Nature* 425:686–91
64. Simpson JC, Wellenreuther R, Poustka A, Pepperkok R, Wiemann S. 2000. Systematic subcellular localization of novel proteins identified by large-scale cDNA sequencing. *EMBO Rep.* 1(3):287–92
65. Shcherbakova DM, Sengupta P, Lippincott-Schwartz J, Verkhusha VV. 2014. Photocontrollable fluorescent proteins for superresolution imaging. *Annu. Rev. Biophys.* 43:303–29
66. van de Linde S, Sauer M. 2014. How to switch a fluorophore: from undesired blinking to controlled photoswitching. *Chem. Soc. Rev.* 43(4):1076–87
67. Knop M, Edgar BA. 2014. Tracking protein turnover and degradation by microscopy: photo-switchable versus time-encoded fluorescent proteins. *Open Biol.* 4(4):140002
68. Pletnev S, Subach FV, Dauter Z, Wlodawer A, Verkhusha VV. 2010. Understanding blue-to-red conversion in monomeric fluorescent timers and hydrolytic degradation of their chromophores. *J. Am. Chem. Soc.* 132(7):2243–53
69. Khmelinskii A, Keller PJ, Bartosik A, Meurer M, Barry JD, et al. 2012. Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nat. Biotechnol.* 30(7):708–14
70. Khmelinskii A, Meurer M, Ho C-T, Besenbeck B, Füller J, et al. 2016. Incomplete proteasomal degradation of green fluorescent proteins in the context of tandem fluorescent protein timers. *Mol. Biol. Cell* 27(2):360–70
71. Mattiazzi Usaj M, Styles EB, Verster AJ, Friesen H, Boone C, Andrews BJ. 2016. High-content screening for quantitative cell biology. *Trends Cell Biol.* 26(8):598–611
72. Newman JRS, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, et al. 2006. Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature* 441:840–46
73. Chong YT, Koh JLY, Friesen H, Duffy K, Cox MJ, et al. 2015. Yeast proteome dynamics from single cell imaging and automated analysis. *Cell* 161(6):1413–24
74. Mazumder A, Pesudo LQ, McRee S, Bathe M, Samson LD. 2013. Genome-wide single-cell-level screen for protein abundance and localization changes in response to DNA damage in *S. cerevisiae*. *Nucleic Acids Res.* 41(20):9310–24

75. Barteneva NS, Fasler-Kan E, Vorobjev IA. 2012. Imaging flow cytometry: coping with heterogeneity in biological systems. *J. Histochem. Cytochem.* 60(10):723–33
76. Krutzik PO, Clutter MR, Trejo A, Nolan GP. 2011. Fluorescent cell barcoding for multiplex flow cytometry. *Curr. Protoc. Cytom.* 6:Unit 6.31
77. Torres NP, Ho B, Brown GW. 2016. High-throughput fluorescence microscopic analysis of protein abundance and localization in budding yeast. *Crit. Rev. Biochem. Mol. Biol.* 51(2):110–19
78. Wachsmuth M, Conrad C, Bulkescher J, Koch B, Mahen R, et al. 2015. High-throughput fluorescence correlation spectroscopy enables analysis of proteome dynamics in living cells. *Nat. Biotechnol.* 33(4):384–89
79. Snijder B, Sacher R, Rämö P, Damm E-M, Liberali P, Pelkmans L. 2009. Population context determines cell-to-cell variability in endocytosis and virus infection. *Nature* 461:520–23
80. Stracy M, Uphoff S Garza de Leon F, Kapanidis AN. 2014. In vivo single-molecule imaging of bacterial DNA replication, transcription, and repair. *FEBS Lett.* 588(19):3585–94
81. Chao JA, Yoon YJ, Singer RH. 2012. Imaging translation in single cells using fluorescent microscopy. *Cold Spring Harb. Perspect. Biol.* 4(11):1–12
82. Uphoff S, Kapanidis AN. 2014. Studying the organization of DNA repair by single-cell and single-molecule imaging. *DNA Repair* 20:32–40
83. Femino AM. 1998. Visualization of single RNA transcripts in situ. *Science* 280:585–90
84. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. 2008. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* 5(10):877–79
85. Xiao L, Guo J. 2015. Multiplexed single-cell in situ RNA analysis by reiterative hybridization. *Anal. Methods* 7(17):7290–95
86. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. 2015. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 348:aaa6090
87. Nelles DA, Fang MY, O'Connell MR, Xu JL, Markmiller SJ, et al. 2016. Programmable RNA tracking in live cells with CRISPR/Cas9. *Cell* 165(2):488–96
88. Tyagi S. 2009. Imaging intracellular RNA distribution and dynamics in living cells. *Nat. Methods* 6(5):331–38
89. Seferos DS, Giljohann DA, Hill HD, Prigodich AE, Mirkin CA. 2007. Nano-flares: probes for transfection and mRNA detection in living cells. *J. Am. Chem. Soc.* 129(50):15477–79
90. Babendure JR, Adams SR, Tsien RY. 2003. Aptamers switch on fluorescence of triphenylmethane dyes. *J. Am. Chem. Soc.* 125(48):14716–17
91. Constantin TP, Silva GL, Robertson KL, Hamilton TP, Fague K, et al. 2008. Synthesis of new fluorogenic cyanine dyes and incorporation into RNA fluoromodules. *Org. Lett.* 10(8):1561–64
92. Paige JS, Wu KY, Jaffrey SR. 2011. RNA mimics of green fluorescent protein. *Science* 333:642–46
93. Filonov GS, Moon JD, Svensen N, Jaffrey SR. 2014. Broccoli: rapid selection of an RNA mimic of green fluorescent protein by fluorescence-based selection and directed evolution. *J. Am. Chem. Soc.* 136(46):16299–308
94. Autour A, Westhof E, Ryckelynck M. 2016. ISpinach: a fluorogenic RNA aptamer optimized for in vitro applications. *Nucleic Acids Res.* 44(6):gkw083
95. You M, Jaffrey SR. 2015. Structure and mechanism of RNA mimics of green fluorescent protein. *Annu. Rev. Biophys.* 44:187–206
96. Buxbaum AR, Haimovich G, Singer RH. 2014. In the right place at the right time: visualizing and understanding mRNA localization. *Nat. Rev. Mol. Cell Biol.* 16(2):95–109
97. Garcia JF, Parker R. 2015. MS2 coat proteins bound to yeast mRNAs block 5′ to 3′ degradation and trap mRNA decay products: implications for the localization of mRNAs by MS2-MCP system. *RNA* 21(8):1393–95
98. Wu B, Chen J, Singer RH. 2014. Background free imaging of single mRNAs in live cells using split fluorescent proteins. *Sci. Rep.* 4:3615
99. Ozawa T, Natori Y, Sato M, Umezawa Y. 2007. Imaging dynamics of endogenous mitochondrial RNA in single living cells. *Nat. Methods* 4(5):413–19
100. O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA. 2014. Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516(7530):263–66

101. Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, et al. 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155(7):1479–91
102. Collins AR. 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.* 26(3):249–61
103. Glei M, Hovhannisyan G, Pool-Zobel BL. 2009. Use of Comet-FISH in the study of DNA damage and repair: Review. *Mutat. Res.* 681(1):33–43
104. Condie AG, Yan Y, Gerson SL, Wang Y. 2015. A fluorescent probe to measure DNA damage and repair. *PLOS ONE* 10(8):e0131330
105. Toga T, Kuraoka I, Watanabe S, Nakano E, Takeuchi S, et al. 2014. Fluorescence detection of cellular nucleotide excision repair of damaged DNA. *Sci. Rep.* 4:5578
106. Tkach JM, Yimit A, Lee AY, Riffle M, Costanzo M, et al. 2012. Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress. *Nat. Cell Biol.* 14(9):966–76
107. Chan J, Dodani SC, Chang CJ. 2012. Reaction-based small-molecule fluorescent probes for chemoselective bioimaging. *Nat. Chem.* 4(12):973–84
108. Purvis JE, Lahav G. 2013. Encoding and decoding cellular information through signaling dynamics. *Cell* 152(5):945–56
109. Newman RH, Fosbrink MD, Zhang J. 2011. Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chem. Rev.* 111(5):3614–66
110. Eggeling L, Bott M, Marienhagen J. 2015. Novel screening methods–biosensors. *Curr. Opin. Biotechnol.* 35:30–36
111. Carter KP, Young AM, Palmer AE. 2014. Fluorescent sensors for measuring metal ions in living systems. *Chem. Rev.* 114(8):4564–601
112. Strack RL, Jaffrey SR. 2013. New approaches for sensing metabolites and proteins in live cells using RNA. *Curr. Opin. Chem. Biol.* 17(4):651–55
113. Rose T, Goltstein PM, Portugues R, Griesbeck O. 2014. Putting a finishing touch on GECIs. *Front. Mol. Neurosci.* 7:88
114. Lock JT, Parker I, Smith IF. 2015. A comparison of fluorescent Ca^{2+} indicators for imaging local Ca^{2+} signals in cultured cells. *Cell Calcium* 58(6):638–48
115. Broussard GJ, Liang R, Tian L. 2014. Monitoring activity in neural circuits with genetically encoded indicators. *Front. Mol. Neurosci.* 7:97
116. Palmer AE, Qin Y, Park JG, McCombs JE. 2011. Design and application of genetically encoded biosensors. *Trends Biotechnol.* 29(3):144–52
117. Akerboom J, Rivera JDV, Guilbe MMR, Malavé ECA, Hernandez HH, et al. 2009. Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. *J. Biol. Chem.* 284(10):6455–64
118. Wang Q, Shui B, Kotlikoff MI, Sondermann H. 2008. Structural basis for calcium sensing by GCaMP2. *Structure* 16(12):1817–27
119. Wardill TJ, Chen T-W, Schreiter ER, Hasseman JP, Tsegaye G, et al. 2013. A neuron-based screening platform for optimizing genetically-encoded calcium indicators. *PLOS ONE* 8(10):e77728
120. Chen T-W, Wardill TJ, Sun Y, Pulver SR, Renninger SL, et al. 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499:295–300
121. Thestrup T, Litzlbauer J, Bartholomäus I, Mues M, Russo L, et al. 2014. Optimized ratiometric calcium sensors for functional *in vivo* imaging of neurons and T lymphocytes. *Nat. Methods* 11(2):175–82
122. Dana H, Mohar B, Sun Y, Narayan S, Gordus A, et al. 2016. Sensitive red protein calcium indicators for imaging neural activity. *eLife* 5:e12727
123. Lohse MJ, Nuber S, Hoffmann C. 2012. Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol. Rev.* 64(2):299–336
124. Sridharan R, Zuber J, Connelly SM, Mathew E, Dumont ME. 2014. Fluorescent approaches for understanding interactions of ligands with G protein coupled receptors. *Biochim. Biophys. Acta.* 1838(1 Pt. A):15–33
125. Prével C, Pellerano M, Van TNN, Morris MC. 2014. Fluorescent biosensors for high throughput screening of protein kinase inhibitors. *Biotechnol. J.* 9(2):253–65

126. Regot S, Hughey JJ, Bajar BT, Carrasco S, Covert MW. 2014. High-sensitivity measurements of multiple kinase activities in live single cells. *Cell* 157(7):1724–34
127. Sakabe M, Asanuma D, Kamiya M, Iwatate RJ, Hanaoka K, et al. 2013. Rational design of highly sensitive fluorescence probes for protease and glycosidase based on precisely controlled spirocyclization. *J. Am. Chem. Soc.* 135(1):409–14
128. Sanman LE, Bogyo M. 2014. Activity-based profiling of proteases. *Annu. Rev. Biochem.* 83:249–73
129. Hertel F, Zhang J. 2014. Monitoring of post-translational modification dynamics with genetically encoded fluorescent reporters. *Biopolymers* 101(2):180–87
130. Peterka DS, Takahashi H, Yuste R. 2011. Imaging voltage in neurons. *Neuron* 69(1):9–21
131. Canepari M, Zecevic D, Bernus O, eds. 2015. *Membrane Potential Imaging in the Nervous System and Heart*. New York: Springer
132. Jercog P, Rogerson T, Schnitzer MJ. 2016. Large-scale fluorescence calcium-imaging methods for studies of long-term memory in behaving mammals. *Cold Spring Harb. Perspect. Biol.* 8:a021824
133. St-Pierre F, Chavarha M, Lin MZ. 2015. Designs and sensing mechanisms of genetically encoded fluorescent voltage indicators. *Curr. Opin. Chem. Biol.* 27:31–38
134. Gong Y. 2015. The evolving capabilities of rhodopsin-based genetically encoded voltage indicators. *Curr. Opin. Chem. Biol.* 27:84–89
135. Jung A, Garcia JE, Kim E, Yoon B-J, Baker BJ. 2015. Linker length and fusion site composition improve the optical signal of genetically encoded fluorescent voltage sensors. *Neurophotonics* 2(2):021012
136. Shcherbakova DM, Shemetov AA, Kaberniuk AA, Verkhusha VV. 2015. Natural photoreceptors as a source of fluorescent proteins, biosensors, and optogenetic tools. *Annu. Rev. Biochem.* 84:519–50
137. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, et al. 2008. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132(3):487–98
138. Spencer SL, Cappell SD, Tsai F-C, Overton KW, Wang CL, Meyer T. 2013. The proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell* 155(2):369–83
139. Kaur A, Kolanowski JL, New EJ. 2016. Reversible fluorescent probes for biological redox states. *Angew. Chem.* 55(5):1602–13
140. Winterbourn CC. 2014. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochim. Biophys. Acta* 1840(2):730–38
141. Pouvreau S. 2014. Genetically encoded reactive oxygen species (ROS) and redox indicators. *Biotechnol. J.* 9(2):282–93
142. Lukyanov KA, Belousov V V. 2014. Genetically encoded fluorescent redox sensors. *Biochim. Biophys. Acta* 1840(2):745–56
143. Zhao Y, Yang Y. 2015. Profiling metabolic states with genetically encoded fluorescent biosensors for NADH. *Curr. Opin. Biotechnol.* 31:86–92
144. Zhao Y, Hu Q, Cheng F, Su N, Wang A, et al. 2015. Sonar, a highly responsive NAD^+/NADH sensor, allows high-throughput metabolic screening of anti-tumor agents. *Cell Metab.* 21(5):777–89
145. Morikawa TJ, Fujita H, Kitamura A, Horio T, Yamamoto J, et al. 2016. Dependence of fluorescent protein brightness on protein concentration in solution and enhancement of it. *Sci. Rep.* 6:22342
146. Altschuler SJ, Wu LF. 2010. Cellular heterogeneity: do differences make a difference? *Cell* 141(4):559–63
147. Kang J, Hsu C-H, Wu Q, Liu S, Coster AD, et al. 2016. Improving drug discovery with high-content phenotypic screens by systematic selection of reporter cell lines. *Nat. Biotechnol.* 34(1):70–77
148. Davis DM, Purvis JE. 2015. Computational analysis of signaling patterns in single cells. *Semin. Cell Dev. Biol.* 37:35–43
149. Okada H, Ohnuki S, Roncero C, Konopka JB, Ohya Y. 2014. Distinct roles of cell wall biogenesis in yeast morphogenesis as revealed by multivariate analysis of high-dimensional morphometric data. *Mol. Biol. Cell* 25(2):222–33
150. Snijder B, Sacher R, Rämö P, Liberali P, Mench K, et al. 2012. Single-cell analysis of population context advances RNAi screening at multiple levels. *Mol. Syst. Biol.* 8:579
151. Kimura A, Celani A, Nagao H, Stasevich T, Nakamura K. 2015. Estimating cellular parameters through optimization procedures: elementary principles and applications. *Front. Physiol.* 6:60
152. Chenouard N, Smal I, de Chaumont F, Maška M, Sbalzarini IF, et al. 2014. Objective comparison of particle tracking methods. *Nat. Methods* 11(3):281–89

153. Wiesmann V, Franz D, Held C, Münzenmayer C, Palmisano R, Wittenberg T. 2015. Review of free software tools for image analysis of fluorescence cell micrographs. *J. Microsc.* 257(1):39–53
154. Cohen AR. 2014. Extracting meaning from biological imaging data. *Mol. Biol. Cell* 25(22):3470–73
155. Winter MR, Liu M, Monteleone D, Melunis J, Hershberg U, et al. 2015. Computational image analysis reveals intrinsic multigenerational differences between anterior and posterior cerebral cortex neural progenitor cells. *Stem Cell Rep*. 5(4):609–20
156. Horvath P, Wild T, Kutay U, Csucs G. 2011. Machine learning improves the precision and robustness of high-content screens: using nonlinear multiparametric methods to analyze screening results. *J. Biomol. Screen.* 16(9):1059–67
157. Sommer C, Gerlich DW. 2013. Machine learning in cell biology-teaching computers to recognize phenotypes. *J. Cell Sci.* 126(24):5529–39
158. Smith K, Horvath P. 2014. Active learning strategies for phenotypic profiling of high-content screens. *J. Biomol. Screen.* 19(5):685–95
159. Abraham Y, Zhang X, Parker CN. 2014. Multiparametric analysis of screening data: growing beyond the single dimension to infinity and beyond. *J. Biomol. Screen.* 19(5):628–39
160. Wait E, Winter M, Bjornsson C, Kokovay E, Wang Y, et al. 2014. Visualization and correction of automated segmentation, tracking and lineaging from 5-D stem cell image sequences. *BMC Bioinform.* 15(1):328