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Modern fluorescent proteins and imaging technologies to study gene expression, nuclear localization, and dynamics

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Recent developments in reagent design can address problems in single cells that were not previously approachable. We have attempted to foresee what will become possible, and the sorts of biological problems that become tractable with these novel reagents. We have focused on the novel fluorescent proteins that allow convenient multiplexing, and provide for a time-dependent analysis of events in single cells. Methods for fluorescently labeling specific molecules, including endogenously expressed proteins and mRNA have progressed and are now commonly used in a variety of organisms. Finally, sensitive microscopic methods have become more routine practice. This article emphasizes that the time is right to coordinate these approaches for a new initiative on single cell imaging of biological molecules.

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Introduction

Recent advances in fluorescent probes with red-shifted spectra resulted in creation of novel red fluorescent proteins (RFPs) and RFP-based biosensors with enhanced spectral and biochemical characteristics. Reduced autofluorescence, low light scattering, and minimal absorbance at the longer wavelengths make RFPs superior probes for cell, tissue, and whole-body imaging [1]. Moreover, introduction of novel RFPs enables multicolor labeling, intravital imaging, super-resolution microscopy, and provides new pairs for FRET techniques. In this review we focus on novel monomeric RFPs and their application for studying gene expression, nuclear localization, and dynamics using advanced imaging. For properties and applications of green fluorescent proteins (GFPs) and other blue, cyan and yellow fluorescent proteins (FPs) we refer to recent reviews [2,3].

Modern red fluorescent proteins

Modern RFPs, with emission maxima exceeding 560 nm, can be divided into five main groups: conventional and split orange, red and far-red FPs, RFPs with a large Stokes shift (LSS-RFPs), fluorescent timers (FTs), and photoactivatable RFPs (PA-RFPs) (Figure 1). We list the currently recommended FPs of each class and their key spectroscopic properties in Table 1.

Conventional red fluorescent proteins

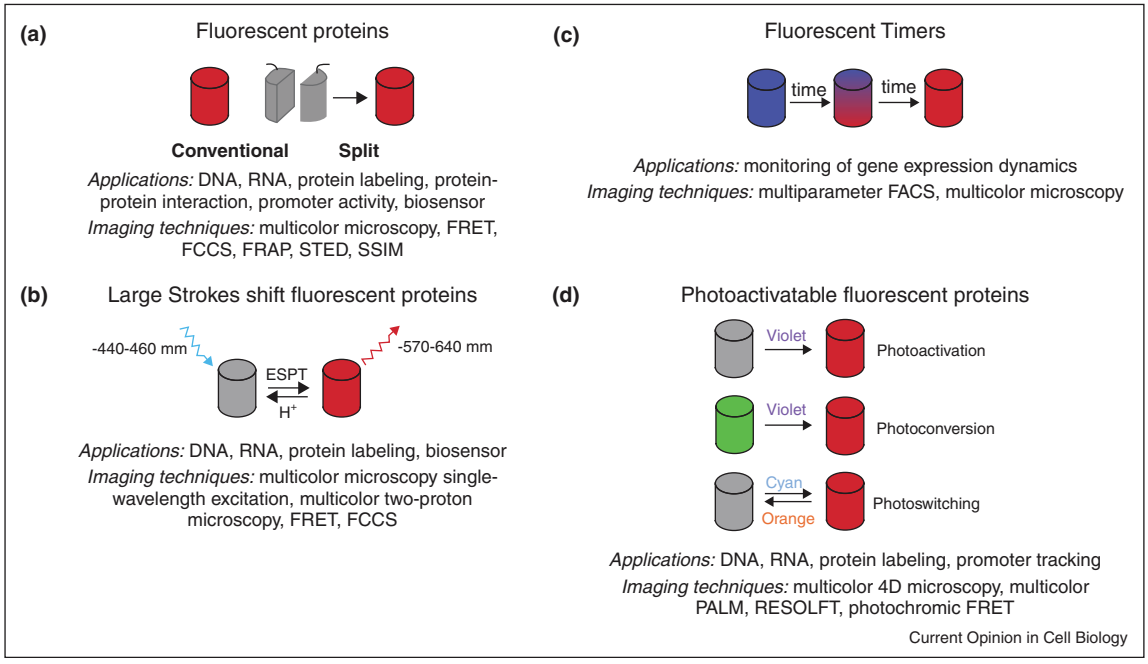
Orange and red FPs

The palette of conventional RFPs have been enriched by the number of enhanced monomeric orange FPs (OFPs) and RFPs for DNA, RNA, and protein labeling in living cells. The novel orange mKOκ [4] and red mRuby [5] FPs are the brightest among the currently available monomeric FPs. High extinction coefficients, pH-stability and extended Stokes shift (47 nm) in case of mRuby make these RFPs attractive as FRET acceptors for yellow donors. However, mKOκ and mRuby are less photostable than mCherry under arc lamp illumination. TagRFP-T and mOrange2, which preserve spectral properties of their precursors TagRFP and mOrange, are attractive for long-term imaging owing to their photostability both under arc lamp and laser illumination, [6]. The improved version of mKate, mKate2, combines brightness and photostability with rapid maturation [7]. Transgenic expression of mKate2 in *Xenopus* embryos revealed reduced cytotoxicity even at high concentration in the cells. Another mKate derivative, split-mLumin, is a novel red bimolecular fluorescent complementation system that shows improved performance in mammalian cells at 37 °C [8].

Far-red FPs

The development of monomeric RFPs with emission beyond 650 nm has recently been achieved. Far-red FPs can be preferable for labeling cellular proteins in strong autofluorescence conditions and for multicolor imaging with OFPs. The TagRFP657 protein, characterized by absorption/emission at 611/657 nm, exhibits low cytotoxicity, high pH-stability and photostability and can be efficiently excited by the standard 633–640 nm red lasers [9]. mNeptune, exhibiting absorption/emission at

Figure 1



Major groups of RFPs, their photophysical properties, and potential applications are shown. **(a)** Conventional and split RFPs. Two non-fluorescent fragments of split FP when brought together form a complete FP barrel. **(b)** Large Stokes shift RFPs. Excited state proton transfer was shown to be responsible for large Stokes shift. **(c)** Fluorescent timers. **(d)** Three types of photoactivatable RFPs. Dark-to-red PAFPs irreversably convert from non-fluorescent state to the fluorescent state under violet light (photoactivation). Green-to-red PAFPs irreversably convert from green fluorescent state to red fluorescent state under violet light (photoconvertion). Red-to-dark photoswitchable FPs reversably convert from non-fluorescent state to the fluorescent state under different lights (photoswitching).

600/650 nm, outperforms TagRFP657 in brightness in mammalian cells [10].

Large Stokes shift fluorescent proteins

Recently, several orange and red FPs with large Stokes shifts (LSS; a difference between excitation and emission maxima more than 100 nm) have been developed on the basis of conventional RFPs [11•]. An excited-state proton transfer (ESPT) occurring upon excitation of a neutral chromophore was shown to be responsible for the LSS observed in these proteins (Figure 1b). The LSS-RFPs are beneficial for imaging under autofluorescence conditions since autofluorescence has a shorter Stokes shift. Moreover, LSS-FPs can be efficiently used with regular FPs for multicolor imaging with a single excitation wavelength and as an additional red color for conventional RFPs. LSSmKate2, optimized for expression in mammalian cells, is recommended owing to its photostability, pH insensitivity and excellent fusion property [12].

Fluorescent timers (FT)

A fluorescent timer changes its color with time owing to a chemical conversion of its chromophore (Figure 1c) [13]. The predictable time course of fluorescence transition allows a quantitative analysis of temporal and spatial molecular events based on the ratio between fluorescence intensities of the two forms. The first monomeric FTs that exhibited distinctive fast, medium, and slow blue-to-red chromophore maturation rates (from around 10 min to 28 h) were developed on the basis of mCherry [14]. The blue and red forms of FTs are bright either alone in protein fusions or together with green FPs for multicolor microscopy. However, noticeable blue-to-red photoactivation of FTs under intense illumination by blue light may complicate their long-term imaging, but still allows efficient application for flow cytometry. Another monomeric FT named Kusabira Green Orange (mK-GO) changes fluorescence from green to orange. The ratio of orange per green fluorescence determined by *in vitro* translation linearly increased and reached a plateau at approximately 10 h [15].

Photoactivatable red fluorescent proteins (PARFP)

PARFPs change fluorescent properties upon irradiation with a certain wavelength. All PARFPs can be divided into the three main groups by color transitions upon illumination (Figure 1d).

Dark-to-red photoactivatable FPs

PAmCherrys [16] and PATagRFP [17••] are non-fluorescent in the dark (non-activated) state, but easily

Table 1

Properties of the modern monomeric red fluorescent proteins

Protein	Ex_{max}, nm	Em_{max}, nm	ε, $M^{-1} \cdot см^{-1}$	QY	Brightness[a]	pK_a	Additional parameter	Ref
Red fluorescent proteins								
							Time[b], h	
mKOκ	551	563	105,000	0.61	64	4.2	1.8	4
mOrange2	549	565	58,000	0.60	35	6.5	4.5	6
TagRFP-T	555	584	81,000	0.41	33	4.6	1.7	6
mRuby	558	605	112,000	0.35	39	5	2.8	5
LSSmKate2	460	605	26,000	0.17	4.5	2.7	2.5	12
mLumin	587	621	70,000	0.46	32	4.7	1.3	8
mKate2	588	633	62,500	0.40	25	5.4	<0.33	7
mNeptune	600	650	67,000	0.20	13	5.4	ND	10
TagRFP657	611	657	34,000	0.10	3.4	5.0	2.0	9
Fluorescent times								
							Time[c], h	
Slow-FT	402	465	33,400	0.35	12	2.6	9.8	14
	583	604	84,200	0.05	4	4.6	28	
Medium-FT	401	464	44,800	0.41	18	2.7	1.2	14
	579	600	73,100	0.08	6	4.7	3.9	
Fast-FT	403	466	49,700	0.30	15	2.8	0.25	14
	583	606	75,300	0.09	7	4.1	7.1	
mK-GO	500	509	35,900	ND	ND	6.0	10	15
	548	561	42,000	ND	ND	4.8		
Photoactivatable red fluorescent proteins								
							Condition[d]	
PAmCherry	564	594	18,000	0.46	8	6.3	Violet	16
PATagRFP	562	595	66,000	0.38	25	5.3	Violet	17
Dendra2	490	507	45,000	0.50	22	6.6	Matures to green	18
	553	573	35,000	0.55	19	6.9	Violet	
mEos2	506	519	56,000	0.84	47	5.6	Matures to green	19
	573	584	46,000	0.66	30	6.4	Violet	
mKikGR	505	515	49,000	0.69	34	ND	Matures to green	20
	580	591	28,000	0.63	18	ND	Violet	
mIrisFP	486	516	47,000	0.54	25	5.4	Violet	21
	546	578	33,000	0.59	19	7.6	Violet / Cyan	
rsTagRFP	440	585	15,300	0.001	0.02	ND	Orange	22
	567	585	36,800	0.11	4	6.6	Blue	

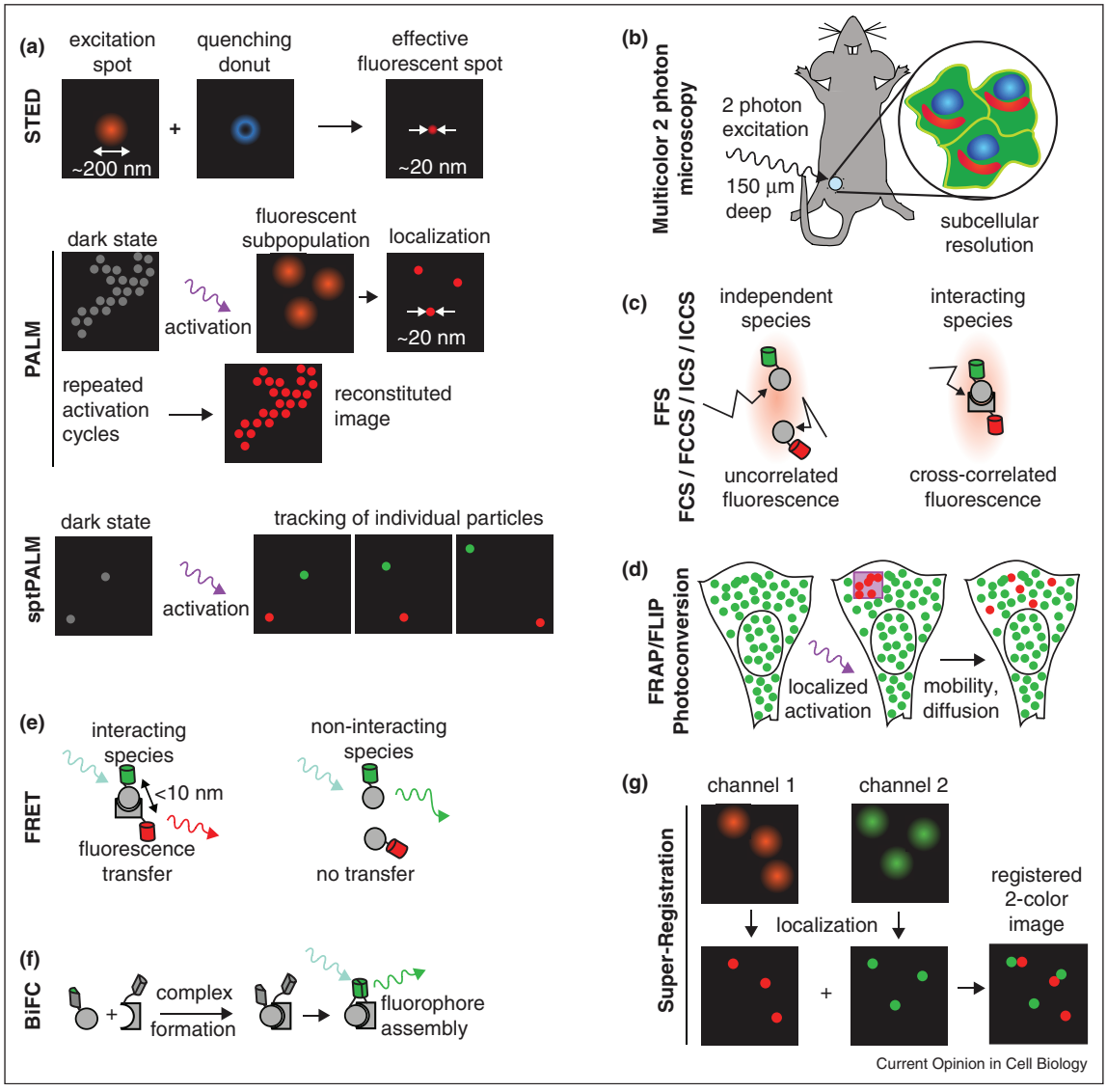
Ex_{max} is the excitation maximum. Em_{max} is the emission maximum. ε is the molar extinction coefficient. QY is the quantum yield.
[a]Fluorescent protein brightness is determined as a product of quantum yield and molar extinction coefficient, divided by 1000.
[b]Maturation half time.
[c]Characteristic time for the color transition.
[d]Condition for the chromophore formation: spontaneous maturation or photoactivation (PAmCherry, PATagRFP), photoconversion (Dendra2, mEos2, mKikGR, mIrisFP), or photoswitching (mIrisFP, rsTagRFP).

undergo irreversible activation under violet light irradiation of relatively low intensity. High photoactivation contrast and photostable red forms make long-term visualization of the activated proteins possible. However, PATagRFP significantly outperforms PAmCherry in pH stability, brightness, and photostability (Table 1).

Green-to-red photoswitchable FPs

All members of this group initially mature to a green-emitting state, which can be irreversably photoconverted into the red fluorescent form upon violet light illumination. The most promising variants of green-to-red PAFPs, which are Dendra2 [18], mEos2 [19], and mKikGR [20], are characterized by high brightness and photostabilities of both fluorescent forms, efficient maturation at 37 °C. Additionally, excellent performance in difficult fusions has already allowed their succesful application for a variety of cell biology problems. It was shown that mKikGR can be also activated by soft radiation of IR laser. A remarkable protein mIrisFP combines properties of photoactivatable and photoswitchable FPs [21]. It undergoes irreversible photoactivation from green to

Figure 2



Advanced microscopy and spectroscopy techniques for imaging gene expression, nuclear localization, and dynamics. **(a)** *Super-resolution microscopy*: The first class of super-resolution microscopy exploits the nonlinear optics to reduce the illumination spot size in technique such as stimulated emission depletion (STED) microscopy, reversible saturable optical fluorescence transition (RESOLFT) microscopy [47], and saturated structured illumination microscopy (SSIM) [48]. The second class involves repeated activation and bleaching of sparsely selected fluorescent molecule and subsequently accurate localization to build up the high resolution images, such as photoactivation localization microscopy (PALM) and its close variants STORM and fPALM [25•]. Single particle tracking PALM (sptPALM) allows tracking of high density molecules in live cell [29]. **(b)** *MPM*: Multiphoton microscopy [49] offers attractive feature over traditional confocal and widefield microscopy for live cell and thick tissue imaging for its increased penetration depth owing to less light scattering, reduced autofluorescence and photobleaching, minimal absorbance of hemoglobin and skin melanin at the longer wavelengths, and its optical sectioning effect. Development of RFPs with large Stokes shift and far-red spectrum enables multicolor *in vivo* MPM with subcellular resolution [12]. **(c)** *FFS*: Fluorescence fluctuation spectroscopy includes a variety of techniques that utilize the fluctuating fluorescence signal when molecules randomly diffuse through a subfemtoliter observation volume created by confocal or two-photon microscope. Fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) [31,50] exploit the temporal decay of correlation/crosscorrelation of the signal to extract the concentration, mobility, and the interaction information. Brightness analysis studies the amplitude of the fluctuation and provides stoichiometry and affinity information of interactions [33]. Image correlation spectroscopy (ICS) and cross correlation spectroscopy (ICCS) [32] measures spatially fluctuating signal from raster-scan laser confocal/two-photon microscopy. They are powerful tools to measure the clustering and dynamics of membrane proteins and receptors. **(d)** *FRAP, FLIP, Photoactivation, Photoconversion*: Molecules in a region of interest are optically highlighted by photobleaching or photoactivation [28]. As the highlighted molecule exchanges with the surrounding unhighlighted ones owing to diffusion and binding, the fluorescence in the ROI is monitored to obtain the kinetic information about mobility and interaction. **(e)** *FRET*: Fluorescence resonance energy transfer measures the effect of excited-state energy transfer from donor to an adjacent acceptor protein. FRET provides evidence for direct interaction since the energy transfer occurs only when donor and acceptor are within 10 nm of each other. Compared with FFS, FRET is independent of the mobility of the molecule under investigation. FRET can be measured simply by acceptor bleaching or

red fluorescent form under violet light, moreover the green and red fluorescent forms can be reversibly switched between dark and fluorescent states by light.

Reversibly photoswitchable RFPs

A small class of photoswitchable RFPs is represented by rsTagRFP [22••]. Initially rsTagRFP matures to a red fluorescent form. However, illumination with blue and yellow light switches the protein into a red fluorescent state or nonfluorescent state, respectively. Switching can be repeated hundreds of times reaching a 20-fold ratio of fluorescence intensities. Thus, rsTagRFP spectral properties are beneficial for sensitive imaging of the switched form.

We have briefly described enhanced versions of FPs from each group that can be used to study problems in cell biology. Following are some applications of these novel RFPs to study gene expression, nuclear localization, and dynamics using advanced imaging techniques.

Microscopy techniques utilizing fluorescent proteins

A variety of microscopy/spectroscopy techniques have been developed in the past decades, which are briefly summarized in Figure 2. Together with FPs, these methods provide key information about cellular function that is otherwise unattainable.

Measuring molecular localization

The localization of molecules within the cell can be followed by 4D microscopy. Time-lapse imaging of FP labeled proteins or mRNAs provides information on their localization and translocation in living cells [23,24]. A new set of methods, termed super-resolution microscopy, have broken the diffraction limit of conventional light microscopy (Figure 2a) [25•]. One form of super-resolution imaging, photoactivation localization microscopy (PALM), involves repeatedly activation and bleaching of sparsely selected fluorescent molecules followed by accurate localization. Introduction of novel photoactivatable RFPs enables multicolor PALM of fixed and living cells [16]. Super-registration microscopy allows co-registration of two spectrally distinct molecules with 20 ms temporal and 26 nm spatial precision in live cells by exploiting a natural cellular marker such as a nuclear pore [26]. A transition from cellular imaging to tissue imaging also become possible with intravital multiphoton microscopy with subcellular resolution [12].

Measuring molecular mobility

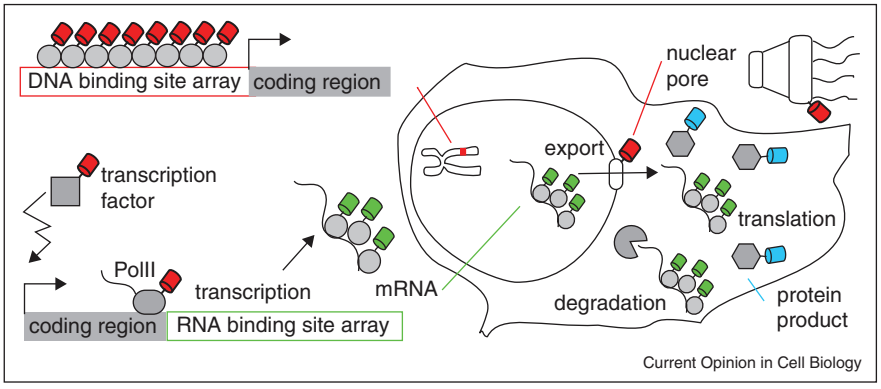
The mobility of molecules can be measured by highlighting a subset of molecules in a small region of interest. This group of techniques includes fluorescence recovery after photobleaching (FRAP), its variation fluorescence loss in photobleaching (FLIP) and reversibly or irreversibly photoactivation of FPs [27,28]. Photoactivation overcomes some limitation of FRAP and FLIP, such as phototoxicity and complex photophysics of some FPs. It enables tracking fast protein movement [28] or even dual-color single particle tracking PALM in live cells [17••,29]. With proper mathematical modeling, these measurements also yield information about binding with the subcellular structure [30•]. Alternative approaches to measure mobility include fluorescence correlation spectroscopy (FCS) [31] and image correlation spectroscopy (ICS) [32]. FCS is able to measure fast dynamics ranging from submicrosecond to second in a specific location. ICS is especially suitable for slower events such as receptors moving on the plasma membrane.

Detecting molecular interactions

An effective way to measure protein–protein interactions in living cells is fluorescence resonance energy transfer (FRET) (reviewed in [33]). Intensity-based ratiometric FRET imaging is easy to implement and widely used to measure fast signaling events of biosensors. Using recently developed photoswitchable rsTagRFP as acceptor and YFP as donor, FRET can be turned on and off, offering an internal control for photochromic FRET (pcFRET) [22••]. FRET, although powerful, suffers from the high false-negative rate to measure protein–protein interactions since it is distance dependent. An alternative approach that is not limited by distance is fluorescence fluctuation spectroscopy (FFS). Brightness analysis in FFS provides straightforward measurements of protein homo-oligomerization [34]. By labeling proteins with different colors, FCCS and ICCS are able to detect interacting species [32,35]. The recently developed hetero-species partition analysis (HSP) utilizes dual-color brightness to measure stoichiometry as well as generate binding curves in living cells [36]. Large Stokes shift proteins provide unique advantages for multicolor FFS

ratiometric imaging. However ratiometric imaging is not appropriate for general purpose protein interaction assays since it depends on relative concentration of donor and acceptor. Fluorescence lifetime imaging microscopy (FLIM) based FRET assay is not limited by this and is commonly applied to detect protein interactions. **(f)** *BiFC* [38]: In bimolecular fluorescence complementation experiment, an FP is split into two segments and fused to two interacting molecules. The two segments remain dark until the interacting partners bring them together and form a complete FP. However, owing to the maturation of fluorophore, there is delay between the interaction and the appearance of fluorescence. In certain scenario, the formation of bimolecular complex is irreversible, which complicates the physiological process understudy. BiFC has been successfully applied to study protein–protein interaction. **(g)** *Super-registration microscopy*: Imaging two interacting molecules in different color with high spatial and temporal resolution is challenging. The super-registration microscopy [26] exploits a natural cellular marker to register positions in different detection channels beyond the diffraction limit. It has been applied to detect a single mRNA particle passing through a single nuclear pore. Currently, the technique is limited to the case that the cellular marker is relative immobile during the time of imaging.

Figure 3



The gene expression in eukaryotic cells involves many steps and numerous components. First transcription requires close cooperation between transcription factor, corregulator, mediator, chromatin remodeler, histone covalent modifier, and basal transcription machinery. After transcription, the mRNA is again subjected to post-transcription modification, export, localization, translation, and degradation. Each individual step can be visualized by tagging corresponding factors with different FPs. Quantitative microscopy techniques allow one to extract dynamic information as reviewed in the text.

experiments since they allow efficient excitation of multiple fluorophores with a single wavelength, eliminating the complications of overlapping lasers and FRET between protein pairs [37•]. Bimolecular fluorescence complementation (BiFC) [38] represents one of the newly developed approaches for visualizing protein–protein interactions. The recently introduced split-mLumin [8] allows simultaneously three-color imaging with a Cerulean and Venus based BiFC system in a single cell.

Imaging gene expression

Gene expression in eukaryotic cells involves many steps and numerous components (Figure 2) [39••,40]. Biochemical studies have identified most players and detailed the enzymatic nature of the process. Various hues of FPs allow multicolor labeling of DNA, RNA, and protein factors involved in gene expression. In addition, novel spectral properties such as photoswitching or fluorescent timers open the way for pulse-chase experiments at a single cell level. Currently it is possible to image three red colors (simultaneous imaging mOrange2 and TagRFP657, and asynchronous imaging of LSS-mKate2). Combination of RFPs with conventional blue/green and large Stokes shift GFPs could image as many as six colors in a single cell.

In Figure 3, we have shown schematically the process of gene expression and how each step can be visualized. First, a specific gene locus on a chromosome can be tagged with DNA binding protein fused to FP by inserting recombinant DNA sequences carrying specific binding sites (such as Lac operator/repressor). Additionally, multiple mRNAs can be visualized in a single cell by incorporating a specific sequence recognized by an RNA binding protein labeled by FPs [41,42]. When the gene is transcribed, multiple nascent transcripts accumulate and illuminate the transcription site.

In order to investigate mechanistic details, various factors that participate or regulate transcription can be labeled. Nuclear receptors (NR) are transcription factors that regulate gene expression in a ligand-dependent manner. Binding of agonist ligand triggers conformation changes of NR that leads to the recruitment of coactivators. Dual-color FFS has been successfully applied to study the concentration, mobility, and interactions of NR and its interaction with coactivators [36]. The transcription dynamics are measured by applying FRAP or photoactivation to the transcription site. In this way, the residence time of various factors and dynamics of RNA polymerase has been measured [43•,44]. It reveals surprisingly dynamic behavior and short binding times for most factors at the transcription site except the polymerase, which elongates the transcript. Novel photoswitching FPs will allow us to follow transcription initiation, elongation, and termination at the same time. MS2 labeled mRNA was tracked in the nucleus and showed that Brownian diffusion dictates the transport [45]. By labeling the nuclear pore complex and applying super-registration microscopy, we and others have observed mRNA going through a single nuclear pore [26,46]. Finally, the mRNA reaches cytoplasm and is translated. Fluorescent protein is commonly used as reporter for gene activity. For example, gene product tagged with fluorescent timers enables monitoring gene expression by conventional microscopy or flow cytometry [4,14].

Conclusions

We are entering a new era of designing probes. These probes have the essential features required for live imaging in cells and tissues: low autofluorescence in the emission spectrum, non-toxic excitation wavelengths amenable to intravital imaging, and timer aspects for following molecules as a function of the biological processes that govern them. The novel reagents can provide

a mix-and-match smorgasbord for an increasing complexity of biological processes to investigate. For instance, illuminating with a single excitation wavelength can now provide four colors of labeled species. The future is bright for researchers searching for biological gold under this rainbow.

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