Chapter 9
Reversibly Switchable Fluorescent Proteins for RESOLFT Nanoscopy

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Abstract Diffraction-limited lens-based optical microscopy fails to discern fluorescent features closer than \(\sim 200\) nm. All super-resolution microscopy (nanoscopy) approaches that fundamentally overcome the diffraction barrier rely on fluorophores that can adopt different states, typically a fluorescent ‘on-’state and a dark, non-fluorescent ‘off-’state. In reversible saturable optical linear fluorescence transitions (RESOLFT) nanoscopy, light is applied to induce transitions between two states and to switch fluorophores on and off at defined spatial coordinates. RESOLFT nanoscopy relies on metastable reversibly switchable fluorophores. Thereby, it is particularly suited for live-cell imaging, because it requires relatively low light levels to overcome the diffraction barrier. Most implementations of RESOLFT nanoscopy utilize reversibly photoswitchable fluorescent proteins (RSFPs), which are derivatives of proteins from the green fluorescent protein (GFP) family. In recent years, analysis of the molecular mechanisms of the switching processes have paved the way to a rational design of new RSFPs with superior characteristics for super-resolution microscopy. In this chapter, we focus on the newly developed RSFPs, the light-driven switching mechanisms and the use of RSFPs for RESOLFT nanoscopy.

9.1 Overcoming the Diffraction Barrier

Optical fluorescence microscopy allows to discern protein distributions in cells. Over the last century, cell biology experienced countless discoveries based on the microscopic visualization of (sub-)cellular structures and their dynamics. Eukaryotic cells, which generally have a diameter of 10–300\(\mu\)m, are relatively small objects crowded with proteins. Hence, conventional optical microscopy, whose resolution is fundamentally limited by diffraction to about 200 nm, inescapably faces the fact that the finest details are not resolvable.
Resolution in microscopy means the separation of distinct features, such as fluorescently labelled proteins. In conventional diffraction limited microscopy, such as widefield (epifluorescence) or confocal laser scanning microscopy, all fluorophores in closer proximity to each other than the diffraction limit are excited together, they emit together, and their emissions diffract together and therefore they are detected together [1]. Hence, with these approaches, structures closer than the diffraction limit are inseparable. The key to fundamentally overcome the diffraction barrier is to render adjacent molecules discernible for a short period of time, preventing different molecules within the same diffraction region from being detected together [2]. This separation can be implemented either in a coordinate-targeted or in a coordinate-stochastic way (for reviews see [1, 3]).

In coordinate-targeted super-resolution microscopy techniques such as RESOLFT nanoscopy, a light pattern is used to induce transitions between two states and to switch fluorophores on and off at defined spatial coordinates. In the simplest scenario, a single beam creating one intensity minimum in a doughnut-shaped pattern is used, but also approaches with several or many minima are possible. At these minima (zeros), there is no off-switching, and the fluorophores in the on-state can fluoresce. These light patterns are scanned over the sample, in order to record a full image. Such scanning approaches require multiple on-off-cycles of the fluorophores.

9.2 RSFPs for Live-Cell RESOLFT Nanoscopy

In its initial definition, the term ‘RESOLFT nanoscopy’ covered all coordinate-targeted nanoscopy approaches relying on two distinct (fluorophore) states including STED nanoscopy [4]. Later, this term was primarily used for coordinate-targeted nanoscopy approaches that rely on metastable fluorophores, most prominently on reversibly (photo-)switchable fluorescent proteins (RSFPs). Compared to other super-resolution microscopy techniques that overcome the diffraction barrier, RESOLFT nanoscopy requires remarkably low light dose to achieve nanoscale resolution. The light intensities used are similar to those applied in live-cell confocal fluorescence microscopy and up to six orders of magnitude lower than those in STED-microscopy. The total light dose deposited on the sample is lower by 3–4 orders of magnitude compared to coordinate-stochastic nanoscopy [5, 6]. As the light intensity is an important factor that determines phototoxicity [7], RESOLFT nanoscopy is particularly suitable for live-cell imaging approaches.

RESOLFT microscopy relies on fluorophores that can be reversibly photoswitched between two metastable states. In the overwhelming majority of applications, these have been reversibly photo switchable fluorescent proteins. RSFPs are structurally highly similar to the green fluorescent protein (GFP). All GFP-based fluorescent proteins share the same overall structure of 11 β-sheets, forming a β-barrel with an α-helix running through the center. The chromophore is autocatalytically formed out of three amino acids within the α-helix requiring only oxygen as an external cofactor for its maturation (Fig. 9.1) [8]. In GFP, the chromophore consists
Fig. 9.1 Structure of EGFP and its intrinsic chromophore. a Side view of the GFP barrel. b Top view of the GFP barrel with the internal chromophore in its center (PDB: 2Y0G). c Structure of the GFP chromophore. The chromophore consists of an imidazolinone and a hydroxyphenyl ring connected by a methine bridge.

of a hydroxyphenyl ring and an imidazolinone ring connected by a methine bridge. By rotation around the methine bridge, the chromophore can adopt either a cis or a trans conformation. The hydroxyphenyl ring can be protonated or deprotonated, thereby shifting the absorption spectrum by about 80–120 nm. The β-barrel shields the chromophore in its center, while numerous noncovalent bonds to surrounding amino acid residues and internal water molecules determine the chromophore position within the protein and its protonation state. The spectral properties of a specific RSFP are a result of the chromophore structure as well as of its interactions with the surrounding residues; therefore, those positions are key targets for mutagenesis to modify the properties of RSFPs.

9.3 Photoswitching Mechanisms of RSFPs

Conventional RSFPs can be classified according to their switching mode, i.e. a ‘positive’ or a ‘negative’ switching mode (Fig. 9.2) [9]. In negative switching RSFPs, the same wavelength that induces fluorescence also switches the RSFP from the on- to the off-state. In contrast, in positive switching RSFPs, the light that induces fluorescence also transfers the protein from the off- to the on-state. Thereby, in conventional RSFPs, switching and fluorescence excitation are directly interconnected. The mechanistic principles of switching have been initially revealed by spectroscopy and crystallography of the RSFPs asFP595 and Dronpa in their respective on- and off-states [10, 11]. In recent years, numerous further studies [12–16], including detailed molecular dynamics studies, ultrafast spectroscopy and crystallography have led to impressive insights into the details of the light driven switching mechanism [17–20]. The mechanistic key event of the switching of all conventional RSFPs analyzed so far is a light induced cis-trans isomerization of the chromophore often combined with a protonation change [13]. This isomerization can be accompanied by shifts of
Fig. 9.2  Schematic overview of the different switching mechanisms. a Negative switching RSFPs can be switched off with light of the wavelength used for fluorescence excitation. b Positive switching RSFPs are switched on by the fluorescence excitation light. c Proteins with decoupled switching are excited with light of a different wavelength than used for on- or off-switching. For each switching mechanism, schematic chromophore structures in the on- and the off-state, examples for the switching wavelengths, and the respective absorption and emission spectra are shown.

The planarity of the chromophore and/or conformational changes of the chromophore pocket and modifications of the hydrogen-bonding network around the chromophore, influencing the chromophores’ ability to fluoresce. Almost all reported crystallographic structures of conventional RSFPs feature a trans-conformation of the chromophore in the off-state and a cis-conformation in the on-state [21]. However, it should be noted, while the majority of fluorescent proteins contain a chromophore in the cis-conformation, bright fluorescent proteins containing a chromophore in the trans-state exist [22]. The only reported RSFP so far with a different molecular switching mechanism is the yellow fluorescent RSFP Dreiklang [23] and its descendant Spoon [24] (Fig. 9.2c). In Dreiklang, fluorescence excitation is decoupled from switching, i.e. one wavelength is used for fluorescence excitation (515 nm), another
for switching on (405 nm), and a third for switching off (355 nm). In Dreiklang, a light-driven reversible hydration/dehydration of the chromophoric imidazolinone ring causes switching by reversibly disrupting the $\pi$-conjugated electron system.

In the following, we will take a closer look at the different switching modes and their application in RESOLFT imaging.

### 9.3.1 Negative Switching Mode

Negative switching RSFPs are most commonly used for RESOLFT nanoscopy. In the dark state, the chromophore is found in the protonated trans-conformation, while in the on-state the chromophore is deprotonated in the cis-conformation (Fig. 9.2a). Excitation of the on-state chromophore results in fluorescence (quantum yield typically 0.1–0.9), switching (quantum efficiency typically in the range of 0.01) [25], or to other non-radiative processes. In comparison, the quantum efficiency for switching the protein from the off- to the on-state is generally substantially higher (quantum efficiency typically in the range of 0.1). The wavelength used for switching the RSFP from the off- into the on-state is usually 80–120 nm shorter than the wavelength for switching off and fluorescence excitation. The precise sequence of the intra-molecular events during the switching process, particularly the sequence of isomerization and protonation change, have been controversially discussed [21]. Recent results for Dronpa and IrisFP conclusively suggested that during the off-to-on transition the chromophore isomerization precedes the protonation change [20, 26].

In single-beam scanning RESOLFT nanoscopy, negative switching RSFPs are generally utilized according to the following imaging scheme: First, the proteins are switched to the on-state with a Gaussian beam, then the proteins in the periphery are switched off with a doughnut-shaped beam, and subsequently the remaining fluorescence in the center (whose size is smaller than the diffraction limit) is read out. The on-switching process has generally a higher quantum efficiency than the off-switching process. Thus, the off-switching step is the most time-consuming step, although fast switching proteins have been developed that reduced the dwell time strongly [5]. However, a too high quantum efficiency for switching off is also not desirable, as switching competes with fluorescence excitation in case of RSFPs with a negative switching mode.

### 9.3.2 Positive Switching Mode

All reported RSFPs with positive switching characteristics, including Padron [9], rsCherry [27] or asFP595 [28], share an Anthozoa heritage. In the off-state, the chromophore of the positive switching RSFPs is generally in the trans-state and deprotonated (Fig. 9.2b). Excitation into the absorption band of the deprotonated
chromophore induces chromophore isomerization to the cis-state. In the cis-state, the protein is in an equilibrium between the protonated and the deprotonated state. Excitation of the protonated cis-chromophore switches the protein to the trans off-state, while excitation of the deprotonated cis-chromophore excites fluorescence. The protonation equilibrium of the chromophore in the cis- and the trans-states is immediately influenced by the amino acids forming the chromophore pocket. Reportedly, the exchange of few amino acids can induce a change in the switching mode of the respective fluorescent protein [9, 12, 27].

Using positive switching RSFPs, the optical system for beam scanning RESOLFT nanoscopy can be reduced to a Gaussian beam for switching on and fluorescence excitation and a doughnut-shaped beam for switching the protein off [29]. A potential advantage of RESOLFT imaging with positive switching fluorescent proteins is that the number of emitted photons during readout is not limited by a competing off-switching process.

9.3.3 Decoupled Switching Mode

The switching of Dreiklang and its descendants differs from the switching of conventional RSFPs by the fact that the excitation of fluorescence does not induce substantial switching [23]. The chromophore of the on-state protein is in an equilibrium between a protonated and a deprotonated state (Fig. 9.2c). Excitation of the deprotonated on-state chromophore at 515 nm results in fluorescence, while excitation of the protonated chromophore at 405 nm leads to a hydration of the C65 atom of the imidazolinone ring, thereby switching the protein to the off-state. More specifically, upon excitation into the absorption band of the protonated chromophore, an ultrafast excited state proton transfer occurs, presumably leading to a charge transfer to the imidizanolinone ring, which subsequently is protonated by Glu222, catalyzing the addition of a water molecule [30]. By this water addition, the π-conjugated electron system is shortened, resulting in the emergence of an absorption band at 395 nm. Excitation of this band results in a water elimination reaction, thereby converting the protein back to the on-state. The positioning of the water molecule reacting with the chromophore and the protonation state of the chromophore are crucial for the efficiency of this switching mechanism. This process is dependent on at least three key amino acids (Gly65, Tyr203, Glu222) [23].

The decoupling of switching from fluorescence excitation enables full control of the state of the protein, which is desirable in RESOLFT nanoscopy. Dreiklang has been used for RESOLFT nanoscopy [23, 31], although it is outperformed by other RSFPs, because some of its other properties (see below) are less favorable for this imaging modality.
9.4 RSFP Properties Important for RESOLFT Nanoscopy

The usefulness of a specific RSFP for live-cell RESOLFT nanoscopy depends on several factors. On the one hand, any usable RSFP must be a suitable fusion tag with a negligible dimerization tendency and a fast maturation rate. On the other hand, it needs to exhibit a combination of favorable photophysical characteristics. The four most important parameters for RESOLFT imaging are the brightness of the protein in its on-state, its switching speed, the residual fluorescence background in the ensemble off-state, and the switching fatigue (Fig. 9.3). These parameters, all of which can be influenced by mutagenesis, are discussed in detail in the following.

9.4.1 Brightness

For a conventional fluorophore, the molecular brightness is defined as the product of the extinction coefficient and the quantum yield. RSFPs can adopt two different states with different quantum yields and in most cases the two states exhibit distinct absorption spectra. Generally, the molecular brightness of the off-state is very small but not necessarily zero. Next to the molecular brightness of the protein in the on-state, two other parameters, namely the effective brightness (of an ensemble of fully matured RSFPs in solution) and the effective cellular brightness (of RSFPs in a living cell) are used to describe and compare RSFPs. In a switching curve (Fig. 9.3a), the effective brightness is measured as the fluorescence intensity of the protein ensemble when switched fully into the on-state. In absolute terms, it is the average molecular

![Fig. 9.3 Important switching parameters. Exemplary switching curve of negative switching RSFPs when switched consecutively on and off with light of different wavelengths. Plotted is the emitted fluorescence intensity. Four key parameters can be determined from these switching curves. a effective brightness of the RSFP in the on-state. b Switching speed. c Residual fluorescence in the off-state. d Switching fatigue](image-url)
brightness of the on-switched protein solution. As a RSFP solution may adopt an 
equilibrium between the on- and the off-state [9, 27], the effective brightness can 
differ drastically from the brightness at equilibrium.

The effective cellular brightness of a specific RSFP is influenced by a number of 
factors, such as its maturation time, its turnover-rate, its expression rate, its usability 
as a tag, temperature, local pH and other difficult to pinpoint factors. As RSFPs are 
primarily utilized for in vivo imaging, the effective cellular brightness is a very crucial 
parameter when RSFPs are compared. The cellular brightness can vary strongly 
between different experimental settings [32, 33] and therefore the data from different 
studies are difficult to compare.

### 9.4.2 Ensemble Switching Speed

The switching speed of an ensemble of RSFPs is typically in the microsecond to sec-
ond range, depending on the light intensity applied [5, 25, 34, 35]. Since RESOLFT 
is an ensemble method, the ensemble switching speed is a key parameter to describe 
and compare RSFPs. In a switching curve (Fig. 9.3), this parameter describes the 
time needed to switch the ensemble from the maximal fluorescence intensity to the 
minimal fluorescence intensity (from the on- to the off-state) or vice versa (Fig. 9.3b). 
The speed is dependent on the applied light intensities and wavelengths; applying 
higher light intensities results in faster switching. As switching is a one photon pro-
cess in both directions [36, 37], the speed and the applied light intensity for the 
switching are largely linearly correlated until saturation of the process sets in. The 
ensemble switching speed is determined primarily by the quantum efficiency for 
switching, by (often ill-defined) intermediate states, and by a crosstalk between the 
on- and the off-switching processes. While high quantum efficiencies for switching 
are beneficial for RESOLFT imaging by shortening the time consuming switching 
step, the connection between switching and fluorescence readout limits the number 
of collected photons in a single switching cycle. For point-scanning RESOLFT in 
living cells, the switching times are generally below 1 ms [5, 6], while in parallelized 
RESOLFT schemes, switching times of tens of milliseconds are suitable [34, 38].

### 9.4.3 Residual Fluorescence in the Off-State

The residual fluorescence in the off-state describes the percentage of the fluores-
cence signal from the off-state compared to the fluorescence signal of the on-state 
(Fig. 9.3c). The switching contrast is defined as the ratio between the fluorescence 
signal of the on-state and the fluorescence signal of the off-state measured on an 
ensemble of proteins.
The switching contrast is determined by the crosstalk between on- and off-switching and the molecular brightness of the on- and the off-state. Furthermore, fast thermal relaxation of the switched protein to the respective equilibrium state, as well as the population of intermediate states, can affect the reachable switching contrast. RSFPs useful for RESOLFT nanoscopy exhibit a contrast higher than 10 (a residual fluorescence below 10%), although smaller values of the residual fluorescence have been reported and are beneficial [25, 39].

9.4.4 Switching Fatigue

With every full switching cycle of an ensemble of RSFPs, a fraction of the proteins is destroyed. This fraction is generally described as the switching fatigue (given as percentage of the effective brightness) (Fig. 9.3d). Presumably, the switching fatigue is mechanistically related to the photostability of a fluorescent protein, which describes its stability while the protein is maintained and excited in the fluorescent on-state.

A low switching fatigue is critical for the usability of RSFPs in RESOLFT microscopy [40]. Photobleaching is highly dependent on the light intensity, as different intensity regimes of photobleaching and nonlinear effects of increasing light intensities have been reported [32, 41]. Therefore, lower light intensities typically result in reduced switching fatigue [39]. Very photostable RSFPs can be switched thousands of times before they are bleached to 50% of their initial brightness [5, 42].

Brightness, switching speed, contrast and switching fatigue are strongly intertwined. The introduction of switching into a conventional fluorescent protein as well as the increase of the switching speed of an existing RSFP by mutagenesis generally lead to a reduction in the fluorescence quantum yield [5, 39, 43, 44]. Furthermore, for negative switching RSFPs a correlation of the switching fatigue with the off-switching speed has been reported [25].

9.5 Overview of RSFPs for RESOLFT Nanoscopy

In the last decade, a number of new RSFPs have been engineered using semi-rational design strategies guided by the crystal structures and insights into the switching mechanism. Thereby, either conventional fluorescent proteins were made switchable, or the characteristics of existing RSFPs were modified. The members of this growing family of RSFPs have been used for a number of applications [45], including intracellular protein tracking [46], photochromic FRET [47], optogenetics [48], optoacoustics [49], and several super-resolution microscopy techniques including coordinate-stochastic approaches [9], SOFI [50, 51], (protected) STED [52, 53] and RESOLFT/NL-SIM [39, 42].

For each application, distinct RSFP properties are crucial and RSFPs have been optimized and adapted for the specific demands. In the following, we provide an
9.5.1 RSFPs Emitting in the Green

RSFPs emitting in the green have been engineered based on several well-characterized fluorescent proteins isolated from Hydrozoan and Anthozoan species. The switching wavelengths of these green fluorescent RSFPs are at ~488 and ~405 nm, corresponding to the absorption bands of the deprotonated and the protonated chromophore. The first experimental implementation of live-cell RESOLFT nanoscopy utilized the negative switching rsEGFP [42], which has been tailored for RESOLFT nanoscopy based on the Hydrozoan EGFP [60]. Derived from rsEGFP, a number of other RSFPs were engineered such as families of rsEGFPs [5, 56], rsGreens [25], and rsFolders [14], with further optimized expression or modified switching characteristics for specific applications.

The EGFP derived RSFPs show very good tagging capabilities, are true monomers, and exhibit fast to very fast switching kinetics. Especially the high resistance of
rsEGFP2 to switching fatigue at high and low light intensities makes it the most widely used RSFP in RESOLFT imaging to date.

The very bright Anthozoa protein Dronpa, which is based on the oligomeric protein 22G from Pectiniidae spec., was the first RSFP to be used in live-cell imaging [46]. Dronpa exhibits comparatively slow switching and very high switching fatigue; Dronpa’s crystal structures of the on- and off-state were solved early, guiding the development of models for the switching mechanisms and strategies for semi-rational protein design [10, 44]. Based on Dronpa, several RSFPs with improved switching properties including rsFastLime, Dronpa-M159T [44] and Dronpa-3 [43] were devel-
developed. The increase in switching speed of these mutants was always accompanied by a reduction of their respective molecular brightness; still, Dronpa-M159T is well suited for point-scanning RESOLFT [61].

A single-residue exchange at the position 159 (Met159Tyr) is sufficient to reverse the switching mode of rsFastLime, i.e. this mutation converted the negative switching RSFP rsFastLime to the positive switching rsFastLime-M159Y. Additional mutations led to the engineering of the bright, positive switching RSFP Padron, exhibiting a high switching contrast [9]. Further mutagenesis changed the switching characteristics and photostability of Padron and led to the development of Kohinoor [29] opening the door to live-cell RESOLFT with positive switching RSFPs.

In recent years, several RSFPs have been engineered using photoconvertible fluorescent proteins as scaffolds. Conventional photoconvertible fluorescent proteins are irreversibly switched from one emission maximum (generally green) to another one (generally red). The mutation Phe173Ser was shown to introduce negative switching to the photoconvertible Anthozoa proteins Dendra2 [62] and mEos [63], resulting in the multiphotochrome RSFPs NijiFP [55] and (with additional mutations) mIrisFP [64]. NijiFP and mIrisFP are both photoconvertible and negatively switchable in the red and green form.

All reported green to red photoconvertible fluorescent proteins have a histidine at the first amino acid position of their chromophore, which is essential for their photoconvertibility [65]. Exchanging this histidine by another amino acid residue can transform a photoconvertible protein into a negative switching RSFP. This was reported for the Anthozoa protein mEos2 [66]. By exchanging the His for different amino acids (Cys, Glu, Phe, Leu, Met, Ser, partially in combination with the Phe173Ser mutation), a series of negative switching RSFPs (mGeos-X), with switching properties similar to those of various Dronpa variants, was produced [57]. Likewise, the His62Leu mutation of the protein mEos3.1 [67] (a true monomer and bright mEos2 descendant), resulted in the RSFP Skyelan-NS [39], which exhibits negative switching characteristics with high brightness and excellent switching contrast.

Following the same strategy, exchange of the chromophoric His in the photoconvertible Anthozoa FP mMaple3 [68], in combination with Met168Ala (corresponding to Met159 in Dronpa), resulted in a series of negative switching RSFPs (GMars-X) displaying various switching kinetics beneficial for different RESOLFT applications, in particular for parallelized RESOLFT [35, 54, 69].

9.5.2 RSFPs Emitting in the Yellow

Based on the yellow fluorescent protein Citrine (a mutant of GFP) [70], Dreiklang [23] and its descendant Spoon [24] were developed. As detailed above, in Dreiklang the switching is decoupled from fluorescence readout and this RSFP has been used for single-beam and parallelized RESOLFT imaging [23, 31, 38]. A limitation of Dreiklang is the comparatively low switching speed, the requirement for two UV light wavelengths for switching and its pronounced switching fatigue. However, in
principle, the decoupled switching mechanism should be very beneficial for most RESOLFT applications. Remarkably, no conventional RSFPs have been reported based on a yellow fluorescent protein so far.

9.5.3 RSFPs Emitting in the Red

Even though the positive switching red RSFP asFP595 was the first RSFP used for proof of concept RESOLFT imaging with purified protein solutions [71], its obligate tetramerization and poor performance as a fusion tag hindered its use in live-cell imaging.

Derived from the Anthozoa protein mCherry, the RSFPs rsCherry, rsCherryRev [27] and rsCherryRev1.4 [59] were established. The high photostability and fast switching of rsCherryRev1.4 facilitated point-scanning and parallelized live-cell RESOLFT nanoscopy with this red emitting RSFP [38, 59], but its poor expression in mammalian cells and a tendency for dimerization limit its applicability.

Based on TagRFP [72], the red emitting RSFP rsTagRFP [47] was developed. It shows good contrast and strong changes in the absorption spectra of the on- and off-states. However, a low extinction coefficient and high switching fatigue prevented RESOLFT imaging in living cells. Only recently, a new family of red fluorescent RSFPs based on FusionRed was engineered [34]. The parent protein shows excellent tagging performance and rsFusionRed2 as well as rsFusionRed3 were successfully used in a parallelized RESOLFT approach [34].

In conclusion, a growing family of RSFPs for RESOLFT nanoscopy is available, each member having distinct properties. As many properties of RSFPs are depending on the light intensities used and the (cellular) environment, data recorded in different laboratories may be difficult to compare. Currently, outside the green fluorescence spectral regime, only a few RSFPs are available, underscoring the need for further protein engineering of red and infrared RSFPs.

9.6 Applications of RESOLFT Nanoscopy

Since the first proof of concept demonstration of RESOLFT nanoscopy using purified asFP595 [71], numerous RESOLFT live-cell applications have been reported [45]. It has been used in point-scanning (Fig. 9.5a) [5, 6, 14, 23, 25, 42, 73] as well as in various parallelized implementations (Fig. 9.5b) [34, 35, 38, 54, 56, 69, 74, 75].

Arguably, rsEGFP2 is the most suited protein in the point-scanning mode, as it displays a combination of favorable properties, most prominently its strong resistance against switching fatigue. Amongst others, rsEGFP2 has been used for imaging of fixed cells that had been labeled by a primary antibody against a protein of interest that was detected by a fusion protein consisting of the IgG binding Z-
Fig. 9.5  Examples of RESOLFT imaging of living cells. a Point-scanning RESOLFT microscopy of intermediate filaments. Keratin19-rsEGFP2 expressed in living Ptk2 cells imaged in the confocal (left) and point-scanning RESOLFT mode (middle) [5]. The graphs show fluorescence intensity line profiles taken at sites indicated in the magnification (right). b Parallelized RESOLFT microscopy of intermediate filaments. Keratin19-rsEGFP-N205S expressed in living Ptk2 cells [56]. c Two color RESOLFT imaging. Vimentin-rsCherryRev1.4 and Keratin-rsEGFP2 were imaged in HeLa cells [59]. d Parallelized RESOLFT microscopy using a red RSFP. Living U2OS cells expressing rsFusionRed3-Vimentin [34]. e In vivo RESOLFT microscopy. Time-lapse point-scanning RESOLFT imaging of rsEGFP2-Tubulin facilitates the observation of tubulin dynamics in intact living fly larvae [6]. Images are adapted with permissions from the listed references. Scale bars: a 1 µm; b 10 µm; c 0.25 µm; d 5 µm; e 1 µm

domain of protein A and rsEGFP2 [73]. Live-cell RESOLFT imaging was established on CRISPR/Cas9 edited cell lines expressing rsEGFP2 fusion proteins at endogenous levels [76]. Likewise, rsEGFP2 was used for RESOLFT nanoscopy in living Drosophila melanogaster tissues and even in intact transgenic second instar fly larvae [6] (Fig. 9.5e). Using rsEGFP2, tens of images with a resolution below 50 nm can be taken for small fields of view with a frame rate of a few seconds. To improve specific characteristics, several variants such as rsGreen and rsFolder were gener-
ated and used for RESOLFT nanoscopy [14, 25]. The fast switching Dronpa variant Dronpa-M159T has similar switching kinetics as rsEGFP2, and it has also been used in a few RESOLFT applications [61, 77]. Next to these negative-switching RSFPs, the positive-switching RSFP Kohinoor [29] and the decoupled RSFP Dreiklang were used for cellular RESOLFT imaging [23, 31, 38].

Several strategies have been applied to perform dual color live-cell RESOLFT nanoscopy, including an approach that exploited the different switching speeds and fluorescence lifetimes of two RSFPs [78]. Despite the fact that the red fluorescent proteins are outperformed by the green ones, some studies utilized a red fluorescent RSFP in combination with a green [59] or yellow fluorescent RSFP [38] for dual label RESOLFT imaging (Fig. 9.5c).

Because of the rather long dwell times, single beam scanning RESOLFT nanoscopy of large fields of view is rather slow and recording of a single image may take several tens of seconds. To address this limitation, several parallelization strategies have been successfully implemented. The first realization of parallelized RESOLFT relied on incoherently superimposed orthogonal standing light waves that allowed to image whole cells in a few seconds [56], and other strategies were reported subsequently (Fig. 9.5b, d) [38, 74, 79]. RESOLFT nanoscopy was also implemented in a light sheet approach [80] and has been combined with STED nanoscopy [52].

### 9.6.1 Other Fluorophores for RESOLFT Nanoscopy

Most current realizations of RESOLFT imaging used RSFPs from the GFP family. Only recently, several other photochromic fluorophores have been utilized for RESOLFT-type nanoscopy. This includes rsLov1, an engineered reversibly switchable protein based on the bacterial photoreceptor YtvA from *Bacillus subtilis* that binds flavin mononucleotide as a chromophore [81]. Also, organic fluorophores were successfully used, including carboxylated photoswitchable diarylethenes [82] and Cy3-Alexa647 heterodimers [83]. Currently, however, these novel probes are outperformed by RSFPs based on the GFP backbone, with regard to basically all photophysical parameters important for live-cell RESOLFT nanoscopy.

### 9.7 Outlook

RESOLFT nanoscopy opens up very flexible imaging schemes. For example, the attained resolution can be adjusted so that the resolution is decreased in favor of reduced phototoxicity, or of an increased recording speed, or in favor of the number of images taken before bleaching. This flexibility, in combination with the relatively low light intensities required, makes RESOLFT nanoscopy an exciting option for live-cell applications.
Recent developments enabling a parallelization of the method allowed to overcome the rather low imaging speed of single-beam solutions. Still, the potential of RESOLFT nanoscopy has not been fully exploited as yet. This is mainly due to the lack of suitable RESOLFT probes emitting in the red and infrared spectral regime, which hinders the development of robust multi-color RESOLFT applications. We predict that this gap will be closed in the near future, as a number of promising templates for the development of such probes are emerging [84].

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