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Dynamic contrast for overcoming spectral interferences in fluorescence imaging

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Abstract. Fluorescence has become a ubiquitous observable in biology. Yet it encounters limitations, which may originate from optical interferences such as ambient light, autofluorescence, and spectrally interfering fluorophores. In this review, we first report on dynamic contrast which can overcome these limitations. Then we specifically describe Out-of-Phase Imaging after Optical Modulation (OPIOM), which proved relevant for multiplexed fluorescence imaging even under adverse optical conditions with several optical setups.

Keywords: Fluorescence imaging, fluorescence microscopy, macroscale imaging, fluorescence endomicroscopy, dynamic contrast, reversibly photoswitchable fluorophores

Introduction

Fluorescence exhibits favorable features for imaging in biology:[1] (i) *Sensitivity*. Fluorescence is red-shifted with respect to the excitation wavelength, which facilitates high contrast against a dark field by spectral filtering. Thus fluorescence imaging is highly sensitive; (ii) *Labeling specificity*. An exogenous fluorophore can be introduced for biomolecule labeling, which is often performed by genetic engineering so as to secure exclusive selectivity. However fluorescence suffers from several limitations for sensitive and quantitative imaging. As a general feature of fluorescence imaging, the common labels exhibit rather broad bands of absorption and emission (at least 50 nm for the half-width), which limits the number of distinguishable labels in multiplexed imaging. Beyond 3–4 labels which can be routinely discriminated, building the final image requires

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to process the fluorescence signals at each pixel (e.g. by linear unmixing,[2, 3] phasor analysis,[4, 5, 6, 7, 8]...). Hence state-of-the-art spectral unmixing in living biological samples currently makes possible to distinguish six fluorophores[9] but at significant cost in terms of photon budget and computation time.[2, 10, 11, 12, 13, 14, 15] As more specific limitations associated with imaging biological samples, reflection or light scattering of ambient light, and autofluorescence from endogenous fluorophores may obscure the emission from a targeted fluorescent label.

In fluorescence imaging, the detrimental interference of ambient light is most often eliminated by recording images in dark environments. When the latter conditions cannot be met, subtracting the images recorded under ambient light with and without light exciting fluorescence has been exploited.[16, 17] However this approach has limited interest under a temporally varying lighting environment. Moreover it cannot be used as well if ambient light is too strong as a consequence of the shot noise, which decreases the measurement precision and degrades the signal-to-noise ratio (SNR).[18] Spectral filters are also poorly effective for selectively retrieving the label signal since the spectrum of the light sources often overlaps with the emission spectrum of the common fluorescent labels. An alternative approach to distinguish the fluorescence signal from ambient light is to tailor the excitation light. Hence camera acquisition has been synchronized with a micro to millisecond long pulsed excitation light for fluorescence imaging under room light to sunlight conditions.[19, 20, 21, 22, 23, 24, 25] Lock-in imaging has also been used to distinguish a fluorescence signal from ambient light. Upon applying periodic modulation of the intensity of the excitation light with an angular frequency at which any modulation of the ambient light can be neglected, the modulated component of the fluorescence can be extracted (e.g after time domain Fourier transform), thereby filtering the background up to the daylight condition.[26, 27] Compared to the preceding approach, lock-in imaging benefits from a higher SNR due to frequency-domain noise filtering.

Autofluorescence is another significant concern in biological samples. It originates from endogenous fluorescent molecules found in animals (e.g. NAD(P)H, flavin, and lipopigments, and porphyrins[28, 29, 30, 31, 32, 33]) and in plants (e.g alkaloids, flavonoids and phenolics, ferulic acid,[34, 35, 36, 37, 38, 39, 40] lipofuscins,[41, 42, 43] and chlorophyll[44]). Spanning from the UV to the IR wavelength range,[45, 46, 47, 48, 49, 50] autofluorescence is difficult to circumvent by spectral filtering. In such a perspective, excitation and emission filters with narrow bandpasses[51, 52, 53, 54] as well as fluorescent labels emitting beyond 650 nm[55, 56, 57, 58, 59, 60, 61, 62, 63] are favorable. When the autofluorescent background is spectrally homogeneous, autofluorescence can be alternatively handled as a fluorophore and eliminated by spectral unmixing. However this approach is of limited interest in biological samples, where autofluorescence arises from diverse substances at specific locations.[64, 65]

It is worthy of note that most strategies reported above to overcome the limitations of fluorescence imaging have exploited discrimination of the targeted label in the spectral domain. Since the optimization of the fluorescent labels (cross section for light

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absorption, quantum yield of luminescence, half-width of absorption/emission bands) has essentially reached its physical limits and considering that fluorescence should remain a much favored observable for imaging live cells,[66] it appears attractive to complement the spectral dimension for further discriminating fluorophores.

Strategies of dynamic contrast

Fluorescence emission reports on light absorption and relaxation pathways of an excited state. As such, it contains dynamic information, which can be used for discrimination in the time domain beyond the sole wavelength.

Fluorescence lifetime imaging microscopy (FLIM)

Fluorescence lifetime imaging microscopy (FLIM) has been early introduced to discriminate fluorophores by the distinct lifetimes of their first singlet excited state,[67, 68] which can be extracted with three protocols. In time-correlated single-photon counting (TCSPC), the delay times of arrival of individual emitted photons are measured and the lifetime(s) is(are) retrieved from the histogram plotting the number of photons versus time after (multi)exponential fitting.[69] Alternatively, the photons emitted following a pulse of laser excitation are detected after a series of time delays in order to reconstruct the decay lifetime(s) after analyzing the recorded intensities.[70] Eventually, in contrast to both former protocols exploiting pulsed excitation, FLIM can also be implemented in the frequency domain: the excitation light is modulated and the fluorescence lifetime is retrieved from analyzing the amplitude and phase delay of the modulated fluorescence intensity.[68, 71]

FLIM has been used to discriminate fluorescent emitters from time-independent ambient light in homodyne detection[26] as well as from autofluorescence by exploiting differences of lifetimes. Hence time-gated detection has been used to selectively image GFP and YFP in plant cells by eliminating autofluorescence from the chloroplast.[72] Despite complex multiexponential fluorescence decay,[73, 74] the long fluorescence lifetime of quantum dots has facilitated their straightforward temporal discrimination against cell autofluorescence and scattered excitation light by time-gated measurements and considerably enhanced the imaging sensitivity.[75, 76] However autofluorescence has an average lifetime around 4 ns (autofluorescence exhibits lifetimes ranging from ps to several ns[77, 78, 79, 80]), which overlaps the one of the most common organic fluorophores. Hence long-lived luminophores (e.g. lanthanide-based luminescent probes,[81] azadioxatriangulenium (ADOTA) fluorophores,[82, 83] Ag clusters[84]) have been designed to detect their emission after complete decay of the autofluorescence. FLIM has also been used in multiplexed fluorescence imaging analysis for histopathological identification of different stained tissues.[85] Yet FLIM-based multiplexed imaging has remained limited by the narrow range of lifetimes of the common bright fluorophores currently used in fluorescence imaging (a same few

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nanoseconds) and it has necessitated deconvolutions[86] or the adoption of subtractive schemes.[68]

Discrimination by the lifetimes of photoswitched states

Recently popularized by super-resolution microscopies,[87, 88, 89, 90] reversibly photoswitchable fluorophores (RSFs) provide more opportunities than simple fluorophores for discrimination with dynamic contrast. RSFs can be photoswitched between states of different brightness upon illumination at one or two different wavelengths.[91, 92, 93] Their light-driven photoswitching intervenes over a wide palette of timescales (μs to s), which facilitates RSF discrimination by analyzing the temporal response of their fluorescence to appropriate light variations. Hence several protocols (e.g. transient state imaging microscopy – TRAST,[94, 95, 96] optical lock-in detection – OLID,[97] synchronously amplified fluorescence image recovery – SAFIRE,[98, 99] and out-of-phase imaging after optical modulation – OPIOM[100, 101, 102, 103, 104]) have exploited the time domain for selectively imaging RSFs against a spectrally interfering background, this neither by relying on deconvolution nor on subtraction schemes.

Transient state imaging microscopy (TRAST) has selectively discriminated organic fluorophores by monitoring the dynamics of their long-lived, photo-induced transient dark states.[94, 95, 96] TRAST has been successfully implemented in multiplexed imaging by exploiting the triplet state, which is sensitive to the presence of molecular dioxygen and heavy ions such as iodide. More specifically, fluorophore-filled liposomes containing or not triplet state quenchers have been distinguished thanks to the different lifetimes of their triplet state.[95]

In Optical Lock-In Detection (OLID), a series of light perturbations drives an RSF throughout cycles of photoswitching.[97, 105, 106] The RSF modulated fluorescence signal is selectively retrieved after cross-correlation with a reference waveform (e.g. acquired from an image zone where only the RSF is present, which cancels any interference from the background (e.g. ambient light, autofluorescence, . . . which do not exhibit any fluorescence photoswitching). OLID has been first validated with a small organic RSF (nitrospiropyrone) and with a reversibly photoswitchable fluorescent protein (RSFP),[97] which can reversibly photoswitch between fluorescent and non-fluorescent states.[93, 107] OLID was subsequently implemented with a fluorescent cyanine, which could be photoswitched at high light intensity.[108] Whereas OLID was not linearly related to the RSF concentration in its initial version,[97] it has been revisited to make it quantitative.[109]

Synchronously amplified fluorescence image recovery (SAFIRE) exploits long-lived dark states, which are depopulated under illumination at a secondary wavelength higher than fluorescence emission.[110, 111] At constant primary excitation light, modulation of the secondary light modulates the fluorescence signal without any crosstalk. After time-domain Fourier transformation of the signal from each pixel, SAFIRE can image a targeted RSF upon eliminating autofluorescence and any signal from spectrally

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interfering fluorophores.[99] Initially validated with Ag nanodots[110] and organic fluorophores,[111, 112] SAFIRE was later implemented with RSFPs.[113, 114, 115] It also encountered many developments such as combining SAFIRE and FRET to overcome the limitation of red-shifted illumination for dark state depopulation,[116] extending SAFIRE to simple fluorophores by exploiting stimulated emission,[117] or applying SAFIRE to fluorescence correlation spectroscopy (FCS).[118]

SAFIRE is endowed to overcome the limitations of autofluorescence, ambient light, and the spectral interference of RSFs, which respond more slowly than the targeted one to the optical modulation of the secondary light. Out-of-Phase Imaging after Optical Modulation (OPIOM) has added the phase lag of the modulated signal to the frequency-dependent modulation amplitude exploited by SAFIRE as a further control parameter.[100, 102] As shown below, the OPIOM exhaustive theoretical framework has led to extract analytic conditions for generating optimum band pass filters, which can directly discriminate the individual contributions of several components in RSF mixtures.

Eventually irreversible photoswitching processes have been used for dynamic contrast. In Bleaching-Assisted Multichannel Microscopy (BAMM), the kinetic signature of photobleaching made possible to discriminate up to three spectrally similar fluorophores with an unmixing algorithm.[119] BAMM was successfully applied to organic dyes, autofluorescent biomolecules and fluorescent proteins.[119]

OPIOM in action

Theoretical background

OPIOM relies on time periodic illumination at one wavelength (light intensity $I(t)$ with I_0 average value and ω angular frequency) to govern RSF photoswitching between two states of different brightness (see Fig. 1a).[100, 101] The thermodynamically stable state **1** is photochemically depopulated in favor of the thermodynamically less stable state **2** (rate constant $k_{12}(t) = \sigma_{12}I(t)$) and it is populated back either by photochemical conversion or thermal recovery (rate constant $k_{21}(t) = \sigma_{21}I(t) + k_{21}^\Delta$; σ_{12} and σ_{21} : photoswitching action cross-sections of the RSF, k_{21}^Δ : rate constant for thermal recovery of **1** from **2**). Since the photoswitching rates depend on $I(t)$, periodic light modulation modulates the concentrations of the two RSF states at ω angular frequency but with a phase delay originating from the relaxation time of RSF photoswitching. At steady-state, the amplitudes of the quadrature-delayed component of the concentrations and of fluorescence emission exhibit a resonance when the parameters of light modulation match the kinetic parameters of RSF photoswitching by fulfilling two conditions:

$$I^0 = \frac{k_{21}^\Delta}{\sigma_{12} + \sigma_{21}} \quad (1)$$

$$\omega = 2k_{21}^\Delta \quad (2)$$

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The resonant quadrature-delayed component of the modulated fluorescence has been chosen as the OPIOM signal (see Fig. 1c). It can be simply extracted by a robust time Fourier transform benefiting from lock-in amplification to improve the SNR. Proportional to the RSF concentration, the OPIOM signal can quantitatively discriminate a targeted RSF against another spectrally similar fluorophore or RSF endowed with different photoswitching kinetics.

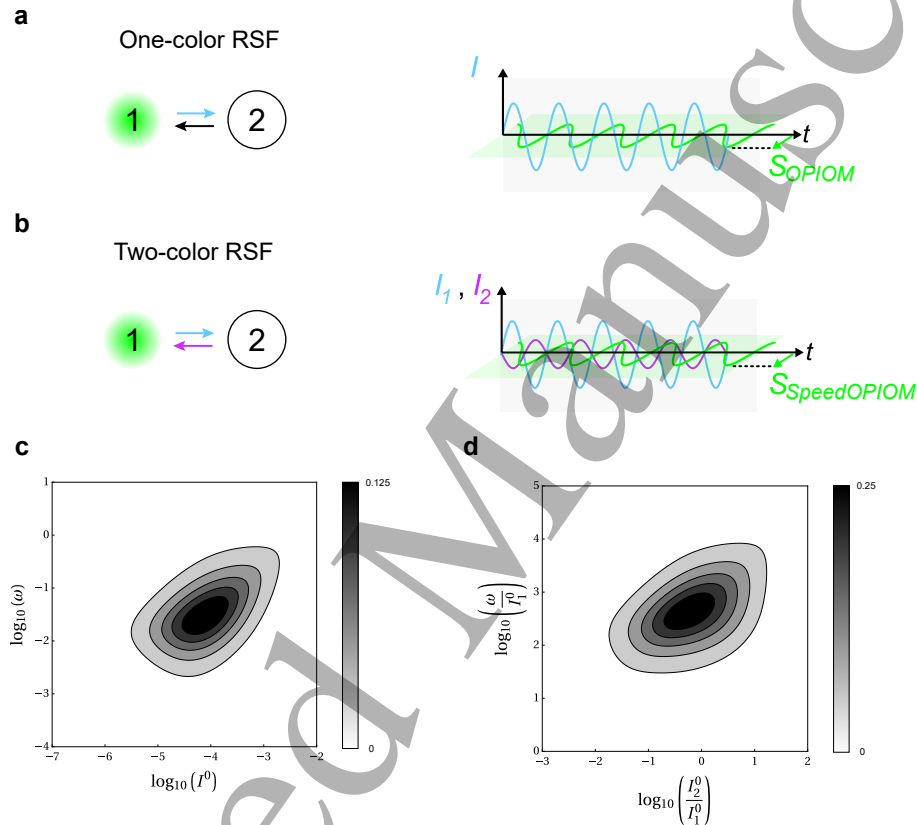


Figure 1. (Speed-) Out-of-phase imaging after optical modulation (OPIOM). (a,c) In OPIOM, a time-periodic light (angular frequency ω and average intensity I^0) modulates the fluorescence emission from an RSF involved in an exchange between a bright and a dark state (respectively **1** and **2**) (a). The OPIOM signal S_{OPIOM} is the amplitude of the out-of-phase component of the fluorescence emission. The OPIOM image of a targeted RSF is selectively and quantitatively retrieved upon adopting resonant illumination parameters I^0 and ω , which match the RSF kinetic parameters σ_{12} , σ_{21} , and k_{21}^{Δ} using Eqs. (1,2) (c); (b,d) In Speed-OPIOM, the time-periodic illumination involves two lights modulated in antiphase at angular frequency ω with respective average intensities I_1^0 and I_2^0 (b). The Speed-OPIOM signal $S_{SpeedOPIOM}$ is the amplitude of the out-of-phase component of the fluorescence emission. It is resonant in the space of the illumination parameters $\{I_2^0/I_1^0, \omega/I_1^0\}$ (d). The Speed-OPIOM image of a targeted RSF is recorded upon matching the illumination parameters I_2^0/I_1^0 and ω/I_1^0 to the RSF kinetic parameters $\sigma_{12,1}$, $\sigma_{21,1}$, $\sigma_{12,2}$, and $\sigma_{21,2}$ using Eqs. (3,4). The panels (c) and (d) display the theoretical plots of the OPIOM and Speed-OPIOM signals normalized by the RSF concentration with $\sigma_{12} = \sigma_{12,1} = 196 \text{ m}^2 \text{ mol}^{-1}$, $\sigma_{21,1} = 0 \text{ m}^2 \text{ mol}^{-1}$, $\sigma_{12,2} = 0 \text{ m}^2 \text{ mol}^{-1}$, $\sigma_{21} = \sigma_{21,2} = 413 \text{ m}^2 \text{ mol}^{-1}$, $k_{21}^{\Delta} = 1.5 \times 10^{-2} \text{ s}^{-1}$ with $I^0 = 100k_{21}^{\Delta}/(\sigma_{12,1} + \sigma_{21,1})$.

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Many RSFs (e.g. green fluorescent RSFPs) can be photoswitched back and forth at two different wavelengths (e.g. 480 and 405 nm with green RSFPs). Thus a secondary light can be applied to accelerate the recovery process of the photoswitched state.[120, 102] In Speed OPIOM which exploits time periodic two-color illumination, the light sources at wavelengths λ_1 and λ_2 are modulated in antiphase at angular frequency ω with respective average light intensities I_1^0 and I_2^0 (see Fig. 1b).[102] Again the Speed OPIOM signal is the quadrature-delayed response of the RSF fluorescence (see Fig. 1d). It exhibits a resonance when the illumination parameters I_2^0/I_1^0 and ω/I_1^0 match the RSF photoswitching cross sections $\sigma_{12,i}$ (respectively $\sigma_{21,i}$) associated with photoswitching from **1** to **2** (respectively from **2** to **1**) driven at λ_i ($i = 1, 2$)

$$(\sigma_{12,1} + \sigma_{21,1}) I_1^0 = (\sigma_{12,2} + \sigma_{21,2}) I_2^0 \quad (3)$$

$$\omega = 2(\sigma_{12,1} + \sigma_{21,1}) I_1^0. \quad (4)$$

Beyond being twice higher than with OPIOM, the Speed OPIOM signal can benefit from higher frequencies of image acquisition. As shown in Eqs.(3,4), increasing I_1^0 and I_2^0 at constant ratio I_2^0/I_1^0 leads to increase ω at resonance.

Implementation

Easily adapted onto commercially available optical setups, the (Speed-)OPIOM imaging protocols can improve their performances without any significant modification. Indeed, they simply require to modulate light sources, which are usually exploited at constant intensity. We have implemented (Speed-)OPIOM on five optical setups: two different fluorescence microscopes,[100, 102], a fluorescence macroscope,[102, 103] and a fluorescence endomicroscope.[104]

OPIOM has been implemented in epifluorescence and single plane imaging microscopies (see Fig. 2a,b).[100] We first used a microdevice filled with RSFP solutions to demonstrate that it selectively gives access to quantitative information on RSFP concentrations. We then applied OPIOM in epifluorescence microscopy to show that it could image RSFPs in fixed and live cells even in the presence of spectrally interfering fluorophores. Finally, we validated OPIOM in single plane imaging microscopy upon evidencing that it could overcome the interfering autofluorescence of tissues and compartments when imaging zebrafish embryos.

Speed-OPIOM has been subsequently implemented in epifluorescence microscopy,[102] macrofluorescence imaging,[102, 103] and fluorescence endomicroscopy[104] (see Fig. 2c–e). In epifluorescence microscopy, we could show that Speed OPIOM can selectively retrieve the fluorescence emission from a targeted RSFP in the presence of spectrally interfering fluorophores and autofluorescence in real time in live cells. Four fluorescent proteins with a similar spectral signature have been discriminated in cells (see Fig. 3a–e). Speed OPIOM was then demonstrated to be relevant for remote imaging with a simple and inexpensive fluorescence macroscope. In a first step, we showed that Speed-OPIOM could enhance the sensitivity and the signal-to-noise ratio for fluorescence detection in blot assays by factors of 50 and 10, respectively, over direct fluorescence

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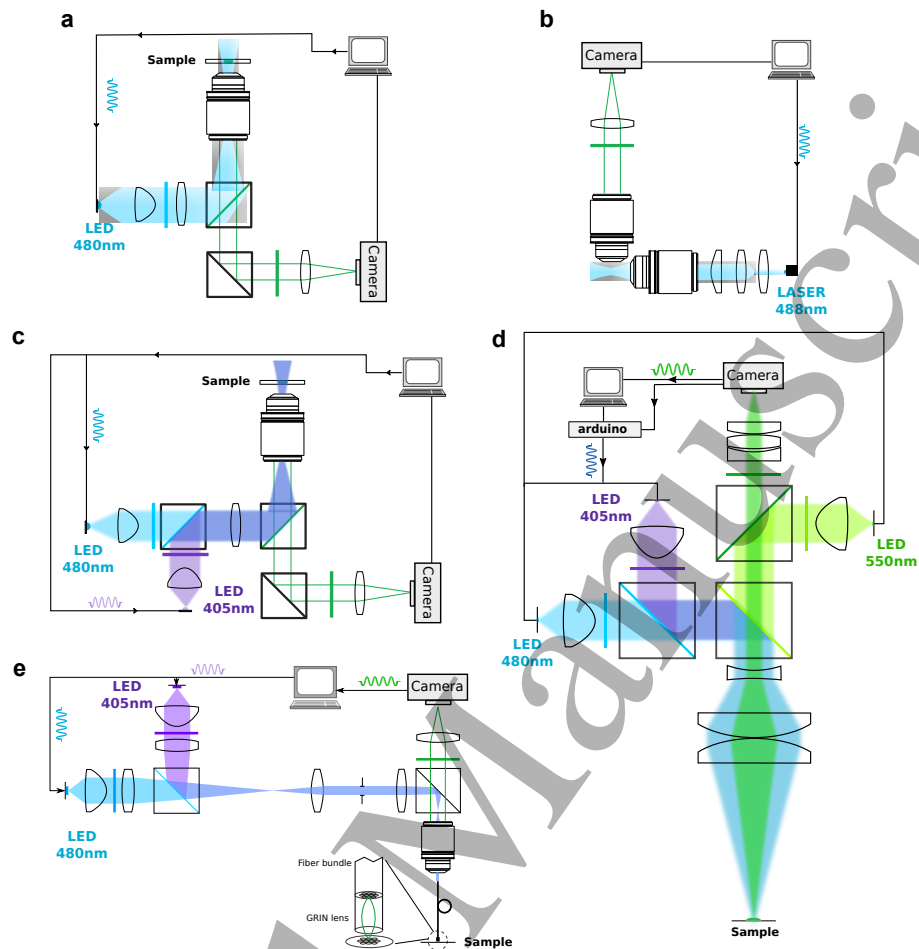


Figure 2. Optical setups for (Speed-)OPIOM implementation. (a) OPIOM in epifluorescence microscopy;[100] (b) OPIOM in single plane illumination microscopy (SPIM);[100] (c) Speed-OPIOM in epifluorescence microscopy;[102] (d) Speed-OPIOM in macroscale fluorescence imaging;[102, 103] (e) Speed-OPIOM in fluorescence endomicroscopy.[104]

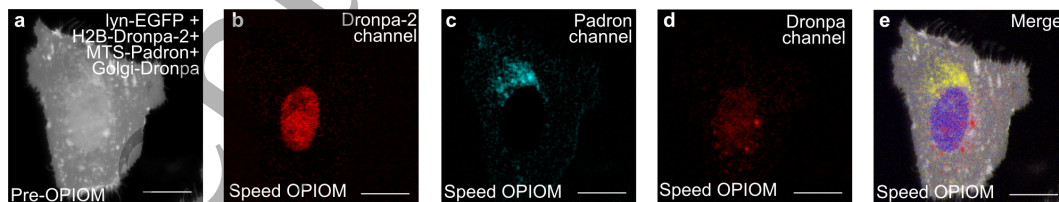


Figure 3. Speed OPIOM can independently image four spectrally similar RSFPs. Pre-OPIOM (a) and Speed OPIOM images tuned to selectively image Dronpa-2 (b), Padron (c), and Dronpa (d). In (e), overlay between the pre-OPIOM image from the Dronpa-2 acquisition and Speed OPIOM images collected in (b-d). Systems: Fixed U2OS cells expressing H2B-Dronpa-2, Mito-Padron, Dronpa-GTS, and Lyn11-EGFP. Localizations: H2B (nucleus), Lyn11 (cell membrane), Mito (mitochondria), GTS (Golgi). The images were recorded at 37°C. Scale bars: 20 μ m. Reproduced with permission from reference [102].

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observation under constant illumination. Then, we evidenced that Speed-OPIOM successfully overcame the strong autofluorescence of growth media that are currently used in microbiology and realized multiplexed fluorescence observation of colonies of spectrally similar fluorescent bacteria. Finally, we easily discriminated fluorescent labels from the autofluorescent and reflective background in labeled leaves, even under the interference of incident light at intensities that are comparable to sunlight. Eventually we have implemented Speed OPIOM in fluorescence endomicroscopy enabling for full-frame acquisition. Using a simple and cheap optical-fiber bundle-based endomicroscope with one-photon excitation integrating modulatable light sources, we first showed that Speed OPIOM is endowed with intrinsic optical sectioning, which restricts the observation of RSFPs at targeted positions within a sample. We also demonstrated that Speed OPIOM efficiently eliminates the interference of the autofluorescence arising from both the fiber bundle and the specimen in several biological samples.

Conclusion

Fluorescence imaging has benefited from significant advances for biological applications, which extend from the development of multiple genetically-encoded fluorophores to the introduction of new microscopies. Overcoming the diffraction limit with super-resolution microscopies has been a first illustration of this impressive progress. More recently, several fluorescence imaging protocols exploiting dynamic contrast (e.g. TRAST, OLID, SAFIRE, OPIOM) have addressed further challenges, which had been considered limiting for fluorescence imaging. In this short review, we more specifically reported on OPIOM. We showed that it is easy to implement on a variety of simple and cheap optical setups, in which it can overcome autofluorescence and ambient light or discriminate several spectrally similar fluorophores. Added to the continuous introduction of RSFPs and genetic techniques for labeling, the imaging protocols exploiting dynamic contrast are expected to open new fields of applications for fluorescence imaging.

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