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Functional super-resolution microscopy of the cell

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Recent advances in super-resolution fluorescence microscopy have led to ~10 nm spatial resolution and profound impact on cell biology and beyond. Here we discuss emerging possibilities to encode functional information of intracellular microenvironments, for example, local pH, small-molecule concentrations, chemical polarity (hydrophobicity), and protein activity, into diverse dimensions of the super-resolution signal, hence a class of approaches we collectively refer to as *functional* super-resolution microscopy. By adding remarkably rich functional information to the already powerful super-resolution arsenal, *functional* super-resolution microscopy transcends the structural information provided by existing methods, and opens up new ways to unveil fascinating local heterogeneities in live cells with nanometer-scale spatial resolution and ultimate sensitivity down to the single-molecule level.

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Introduction

The rapid advances in super-resolution (fluorescence) microscopy (SRM) over the past decade [1,2], including single-molecule localization microscopy (SMLM, for example STORM [3], (F)PALM [4,5], and PAINT [6]) and stimulated emission depletion (STED) microscopy [7,8], have revolutionized how we perceive the world. The exquisite spatial resolution down to ~10 nm, combined with the inherent benefits of fluorescence microscopy, in particular, high molecular specificity and good compatibility with live samples, enables exciting discoveries in biology and beyond [1,2,9].

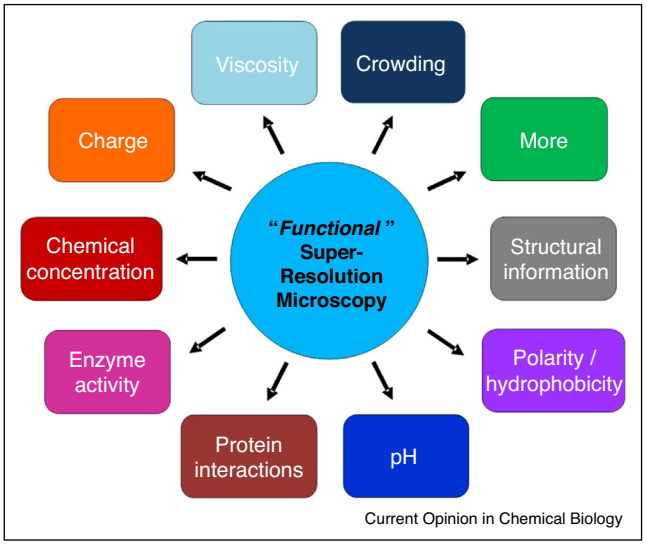
As implied by its name, the primary aim of SRM has been to improve the attainable spatial resolution, namely, to resolve the finest possible ultrastructure. The structural information, however, does not tell the full story of how the cell *functions*. The astoundingly ordered internal organization achieved within the small volume of the cell does not stop at the level of geometrical shapes. Driven far away from equilibrium, the local physicochemical parameters within a cell, for example, pH, small-molecule concentrations, chemical polarity (hydrophobicity), and protein activities, also vary greatly over short distances. Such intracellular 'microenvironments' locally regulate biochemical reactions and other cellular processes, and so dictate cell functions at a fundamental level [10,11]. Would it be possible to unveil and visualize such intracellular *functional* information with nanometer-scale (nanoscale) spatial resolution similar to what has been achieved for *structures* with SRM? If so, what new insights can be gained toward our understanding of how the cell works?

In this review, we discuss emerging new possibilities to answer these fascinating questions by encoding specific *functional* information of intracellular microenvironments into certain, in some cases novel, dimensions of the SRM signal, a class of methodologies we collectively refer to as *functional* SRM (*f*-SRM; Figure 1). Although multifunctional and multiparametric fluorescence microscopy, often enabled by environment-sensitive and chemical-responsive fluorescent probes [11–16], long preceded the development of SRM, the relatively low spatial resolution (~300 nm) achieved with conventional, diffraction-limited microscopy limits how localized the microenvironments can be probed. As the probed volume scales cubically with the linear dimension, a 10-fold enhancement in spatial resolution, which is often achieved in SRM, could reduce the probed volume by 1000 times, hence dramatic reduction of interference from the undesired surrounding signal when compared to the desired local signal. For SMLM-based techniques, the possibility to map local properties by examining the response of each probe molecule one at a time further represent the ultimate sensitivity, effectively removing the interferences between different molecules. The outstanding resolution and sensitivity thus offer exciting potential opportunities to uncover local *functional* information that would otherwise be averaged out with traditional approaches. Below we summarize emerging *f*-SRM efforts that start to demonstrate such possibilities, and we group our discussion by how the functional information is optically encoded.

Fluorescence intensity

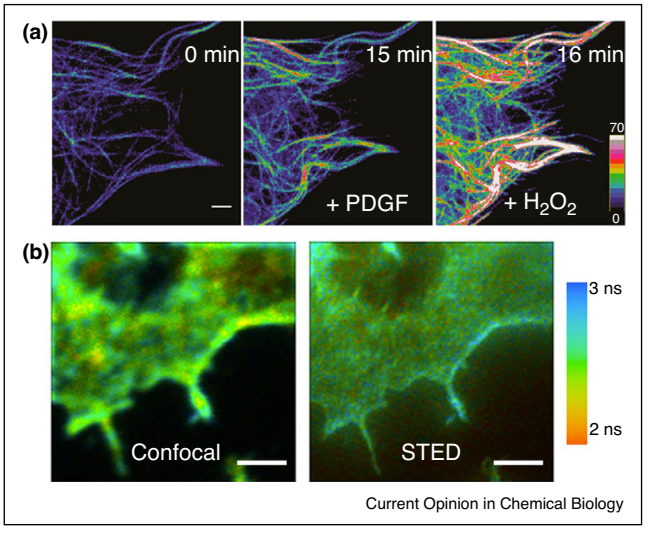
Fluorescence intensity is conceivably one of the most straightforward parameters for encoding functional

Figure 1



Functional super-resolution microscopy (*f*-SRM) explores the possibility to transcend the structural (shape) information offered by existing super-resolution methods and unveil multidimensional information of diverse intracellular *functional* parameters, like those shown in this diagram, with nanoscale spatial resolution and ultimate sensitivity down to single molecules.

Figure 2



f-SRM through fluorescence intensity and lifetime detections. **(a)** STED images of EB3–HyPer2 fluorescence intensity change in an NIH 3T3 cell at indicated time points, after stimulation with 10 ng/mL PDGF and subsequent addition of 200 μM H_2O_2. **(b)** Comparison of confocal (left) and STED (right) fluorescence lifetime images of a fixed *Drosophila* S2 cell with Alexa Fluor 594-phalloidin-labeled actin. Scale bars: 2 μm. Panel (a) is adapted from [17•]. Panel (b) is adapted from [22].

information. Indeed, many fluorescent reporters for chemical imaging are based on fluorescence turn-on [11,13–16].

Mishina *et al.* [17•] adopted this strategy to visualize the concertation of H_2O_2 in live cells with STED SRM. By tagging HyPer2, a fluorescence turn-on fluorescent protein (FP) biosensor for H_2O_2, to the cytoskeleton, STED SRM images provided ~3-fold enhancement in spatial resolution when compared to conventional confocal microscopy. Treatment of the cell with platelet-derived growth factor (PDGF), as well as with H_2O_2, led to modest and substantial increases in the STED-measured HyPer2 intensity (Figure 2a), respectively, attributable to corresponding rises in local intracellular H_2O_2 concentration. In a more sophisticated approach, Mo *et al.* [18•] developed biosensors based on the increased fluctuation in fluorescence intensity as two FPs were brought into proximity, and thus, through stochastic optical fluctuation imaging, showed subdiffraction features of protein kinase A (PKA) activity on the cell plasma membrane.

Fluorescence lifetime

Although the fluorescence intensity is easy to measure, its limitation for reporting functional information is also apparent. The detected intensity depends on the local concentration of the fluorescent probe, and so it is difficult to quantify for absolute values. Interpretation of time-dependent signal changes is further complicated by photobleaching, which could be significant for the strongly illuminated SRM experiments.

Fluorescence-lifetime imaging microscopy (FLIM) [19] is a powerful, intensity-insensitive method for probing local environments. The exponential decay rate of fluorescence emission, typically on the time scale of nanoseconds, depends strongly on both the dye identity and dye-environment interactions, thus a valuable reporter.

For SRM, although FLIM has been successfully incorporated with STED [20–23], the focus has been on the unmixing of different dyes into separate color channels for multi-target SRM [21,23]. Lesoine *et al.* [22] examined Alexa Fluor 594-phalloidin-labeled actin cytoskeleton in fixed cells, and noticed varying local fluorescence lifetime in the STED image (Figure 2b), a result potentially consistent with varied quenching interactions between the tagged dye and the local components of the cell. However, as a rather Gaussian-like distribution was found for the measured lifetime at different locations, it was unclear if the observed local variations merely reflected statistical error [22]. Nonetheless, given the wide usage of FLIM for environment sensing [19], lifetime-resolved SRM stands as a promising direction for future *f*-SRM efforts.

Fluorescence polarization

Fluorescence polarization and anisotropy measurements provide information on the orientation and rotational

mobility of fluorescence molecules [24]. For SRM, polarization-resolved SMLM has been achieved by splitting the fluorescence into polarizations parallel and perpendicular to the excitation laser using either a polarizing beam splitter [25] or a Wollaston prism [26•,27], and by modulating the polarization orientation of the excitation laser [28]. Gould *et al.* [25] thus showed, through polarization FPALM, local heterogeneities in the fluorescence polarization anisotropy of overexpressed Dendra2-actin molecules in fixed fibroblasts. Cruz *et al.* [26•] performed polarization-resolved dSTORM and found certain, but not all, dyes conjugated to phalloidin exhibited restricted polarization orientations when labeled to actin stress fibers in fixed cells. For *in vitro* samples, polarization-resolved SMLM has also demonstrated preferred orientations for dyes labeled to DNA strands [26•,28] and insulin amyloid fibrils [27]. Thus, polarization-resolved SRM provides orientation information of the tagged fluorophores, hence new structural, and potentially functional, insights.

Ratiometric detection of spectral shift

Ratiometric detection is a commonly used, relatively simple strategy to detect the color changes of fluorescence probes. Here, a dichroic mirror splits the fluorescence into long-wavelength and short-wavelength components, respectively, and the relative intensities measured for the two components provide the spectral information.

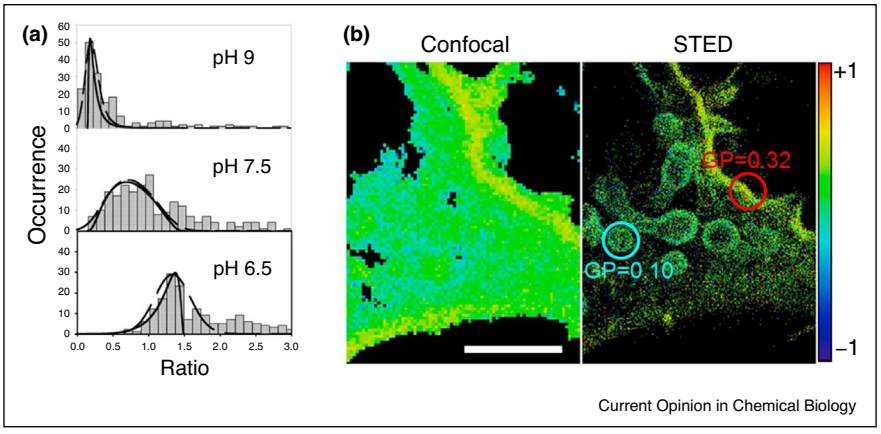
For single-molecule imaging, Brasselet and Moerner [29] investigated the local pH in agarose gels by sparsely doping dextran molecules tagged with SNARF-1, a fluorescent pH indicator. The pH-dependent spectral shift was measured as the ratio of single-molecule emission between two wavelengths centered at 580 and 640 nm. Interestingly, although the mean values from single-molecule ensembles matched that of bulk measurements, histograms of emission ratios of individual molecules at different pH revealed more scattered distributions at intermediate pH (Figure 3a). This difference suggests higher spatial heterogeneity of protonation chemistry at intermediate pH, which could not have been detected with ensemble measurements. Extending related approaches to SMLM-type SRM [31–33] and to cells represents future challenges.

For STED SRM, Sezgin *et al.* [30••] achieved ratiometric spectral detection for several polarity-sensitive, solvatochromic membrane probes. The threefold enhancement of spatial resolution over confocal microscopy was instrumental in revealing local polarity differences. In particular, for Di-4-AN(F)EPPTEA-labeled live cells, STED detected significantly smaller generalized polarization (GP) values, and thus reduced local molecular order, for endocytic vesicles near the plasma membrane, a nanoscale feature that was obscure under diffraction-limited confocal spectral imaging (Figure 3b). Together with the above-discussed STED-FLIM experiments, it appears *f*-SRM through combing environment-sensing dyes with STED could lead to great promises.

Spectrally resolved super-resolution microscopy

The full power of integrating spectrum-changing fluorescent probes with SRM is unleashed by the recent rise of spectrally resolved SMLM (SR-SMLM) [34]. Whereas for conventional fluorescence microscopy, to measure the

Figure 3



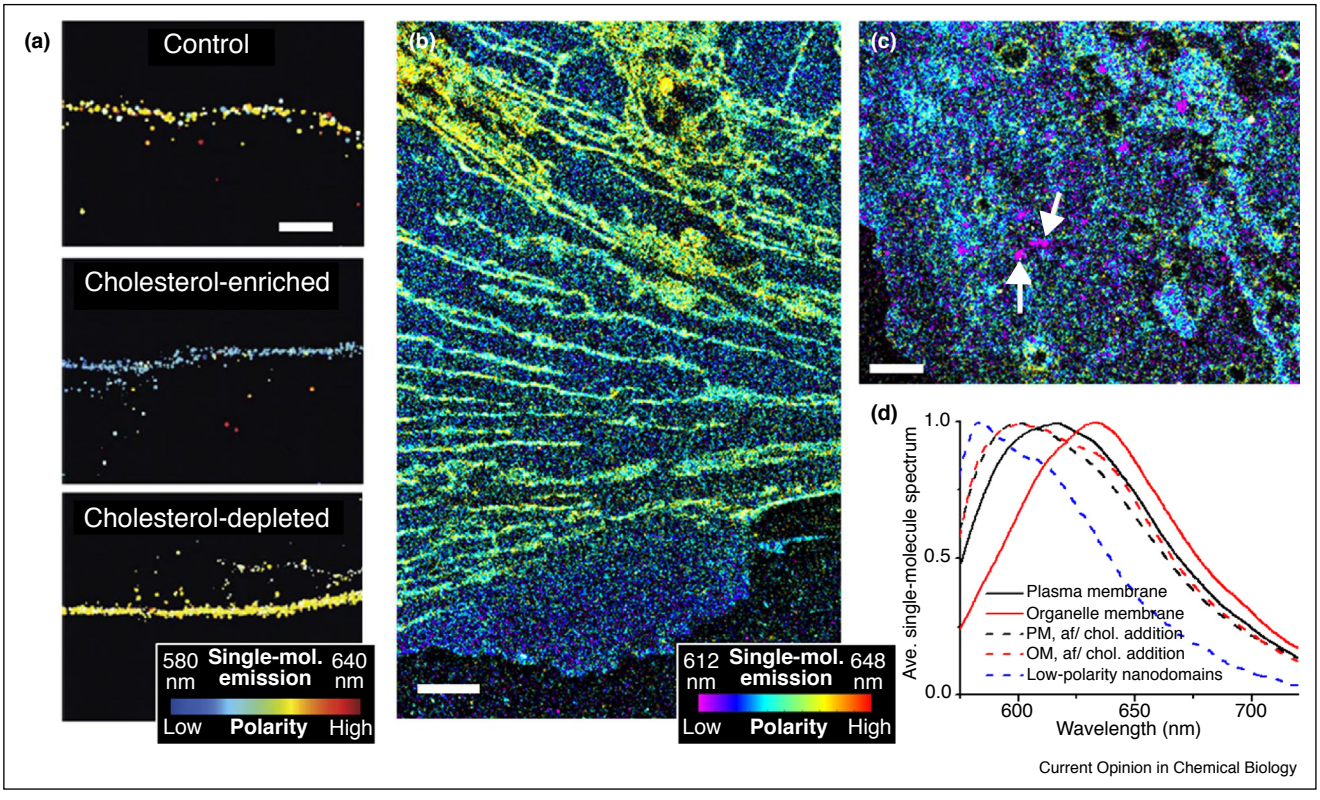
f-SRM through ratiometric color detection. **(a)** Histograms of the emission ratio $R = I_{580\,nm}/I_{640\,nm}$, $I_{580\,nm}$ and $I_{640\,nm}$ being the fluorescence intensities detected at 580 and 640 nm, respectively, for single SNARF-1-dextran molecules immobilized in agarose gels at different pH. **(b)** Ratiometric confocal and STED images of Di-4-AN(F)EPPTEA in a living CHO cell. Color denotes the generalized polarization (GP), $(I_1 - I_2)/(I_1 + I_2)$, where I_1 and I_2 are the fluorescence intensities detected in the 520–570 nm and 620–700 nm channels, respectively. Endocytic vesicles (blue circle) show smaller GP values than the plasma membrane (red circle). Scale bar: 500 nm. Panel (a) is adapted from Ref. [29]. Panel (b) is adapted from Ref. [30••].

actual emission spectra (beyond ratiometric detection) necessitates spatially confined illumination or detection to avoid convolution of the spectral and spatial signal, single fluorescent molecules are, in themselves, self-confined point sources. By dispersing the emission of many single molecules simultaneously in the wide field and implementing fluorescence on–off switching, the spectra of millions of individual molecules can be obtained within minutes [35•]. Assembling such spectral information, together with the super localized positions of the same molecules, thus enables SR-SMLM.

The initial demonstrations of SR-SMLM aimed at multicolor SRM [35•,36,37]. Zhang *et al.* [35•] demonstrated that with spectrally resolved STORM, four dyes only ~10 nm apart in emission wavelength can be distinguished with negligible misidentification when labeled to different targets in fixed mammalian cells. Moreover, excellent three-dimensional (3D) spatial resolution was obtained for every dye, and the obtained 3D SRM images for all dyes were automatically aligned with the same coordinates. The rather narrow distribution of single-molecule spectra in this work contrasted early results of single molecules at solid surfaces [38–41], and indicated that the single-molecule fluorescence of many dyes was insensitive to the local environments in fixed cells. In comparison, Mlodzianoski *et al.* [36] carried out spectrally resolved FPALM, and noticed spectral wandering for a fraction of the detected single-molecule fluorescence of FPs in fixed cells, the mechanism of which remains to be elucidated.

For *f*-SRM, Bongiovanni *et al.* [42••] employed Nile Red, a widely studied solvatochromic dye [44], to enable surface hydrophobicity mapping through grating-based spectrally resolved PAINT. This enabled the probing of surface hydrophobicity for *in vitro* protein aggregates of α-synuclein and amyloid-β. In particular, it was found that the amyloid fibers have different surface hydrophobicity from their constituting oligomers, which may relate to cellular toxicity. For cells, this work visualized the hydrophobicity of cell plasma membranes in a side-view geometry, and noted spectral shifts for cholesterol-enriched and cholesterol-depleted conditions (Figure 4a).

Figure 4



f-SRM through spectrally resolved super-resolution microscopy. **(a)** Grating-based spectrally resolved PAINT image of the side view of the Nile Red-labeled plasma membrane of HEK293 cells, untreated, cholesterol-enriched, or cholesterol-depleted. Redder emission corresponds to higher solvent polarity (lower hydrophobicity). **(b)** Prism-based spectrally resolved PAINT image of the in-plane view of the Nile Red-labeled membrane system of a live PtK2 cell. **(c)** Prism-based spectrally resolved STORM image of the Nile Red-labeled membrane system of a cholesterol-added COS-7 cell. Arrows point to low-polarity nanodomains. **(d)** Averaged single-molecule spectra at the plasma membrane (PM), organelle membrane (OM), and the low-polarity nanodomains. Scale bars: 100 nm (a); 2 μm (b, c). Panel (a) is adapted from Ref. [42••]. Panels (b–d) are adapted from Ref. [43••].

Moon *et al.* [43••] utilized spectrally resolved STORM and spectrally resolved PAINT, in combination with Nile Red staining, to reveal nanoscale heterogeneity in cell membranes. As a prism was used to attain a weaker dispersion for less overlapping of single-molecule spectra, as well as to reduce photon loss, the same strategy adopted in the original work of spectrally resolved STORM [35•], this work enabled *f*-SRM mapping of the local chemical polarity for dense structures as in-plane views of the membrane system of live mammalian cells (Figure 4b). This helped reveal distinct polarity characteristics of the plasma membrane versus the membranes of nanoscale intracellular organelles, with the latter being more polar (less hydrophobic) (Figure 4b, d), in line with their expected biological functions. Cholesterol manipulation further showed that this striking contrast in local chemical polarity was driven by differences in the local cholesterol level. Moreover, *f*-SRM unveiled the formation of low-polarity, raft-like nanodomains ~100 nm in size upon cholesterol addition (Figure 4c, d) or cholera toxin B treatment conditions, thus shedding new light onto a contentious subject [45,46].

Xiang *et al.* [47•] recently showed that *f*-SRM based on spectrally resolved PAINT could be extended to probe the nanoscale structure and composition of organic layers adsorbed on glass surfaces. One striking finding was that mixtures of miscible liquids spontaneously demixed into nanodroplets of varying compositions and sizes on the glass surface. Although this study did not involve cells, it provided new insights into the phase separation behaviors of molecules at the nanoscale, and so could inspire future work on understanding related intracellular processes [48].

Outlook

In conclusion, *f*-SRM has emerged over the past few years as a new class of powerful methodologies to address the highly intriguing, yet highly challenging, *functional* aspects of cell biology at the nanoscale. Diverse physicochemical parameters have been examined, and numerous strategies have been invoked to encode the sought functions, in many cases prompting the development of new optical tools that access new dimensions of the SRM readout. We expect future work to expand on existing possibilities by both encoding untested new functions and by further exploring other possible dimensionalities of the SRM signal (e.g. the emerging dimension of single-molecule velocity and diffusivity [49]), including through correlated approaches that tap into the power of other microscopy and spectroscopy techniques [50]. Throughout such endeavors, the development and/or identification of suitable, environmentally sensitive fluorescent probes undoubtedly hold the key. Thus, the multifunctional and multidimensional approach of *f*-SRM relies on multidisciplinary efforts; its future success demands in-depth discussion and collaboration between researchers across diverse fields.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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