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1 Single-molecule localization microscopy

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24 data deposition (J.G., S.M. and C.Z.), Limitations and optimizations (M.T.G. M.La, F.S., R.J., M.
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26 Primer (C.Z.).
27
28

29 **Abstract**

30 Single-molecule localization microscopy (SMLM) has emerged as a particularly powerful family
31 of imaging techniques that dramatically improve the spatial resolution over standard, diffraction-
32 limited microscopy techniques and can reveal biological structures at the molecular scale. In
33 SMLM, individual fluorescent molecules are computationally localized from diffraction-limited
34 image sequences, and the localizations are used to generate a super-resolution image, or in some
35 cases a super-resolution movie or molecular trajectories. In this Primer, we first introduce the
36 basic principles of SMLM techniques, then describe the main experimental strategies to perform
37 SMLM, including fluorescent labeling, sample preparation, hardware requirements and image
38 acquisition in fixed and live cells. We then explain how low-resolution image sequences are
39 computationally processed to reconstruct super-resolution images and/or extract quantitative
40 information, and highlight a selection of biological discoveries enabled by SMLM and closely
41 related methods. We also discuss some of the main current limitations and potential artifacts of
42 SMLM, as well as ways to alleviate them. Finally, we propose an outlook on advanced techniques
43 and promising new developments in the fast-evolving field of SMLM. We hope that this Primer
44 will be a useful reference for both newcomers and practitioners of SMLM.

45

46

47 [H1] Introduction

48 The spatial resolution of standard optical microscopy is limited to roughly half the wavelength of
49 light¹, as a consequence of the optical phenomenon of diffraction. Because of diffraction, the 'point
50 spread function' (PSF) — i.e., the image of an arbitrarily small source of light through a lens-
51 based microscope — is not a point, but an Airy pattern, with a central peak approximately ~200-
52 300 nm in width (**Fig. 1a**). Diffraction thus leads to a blurring of structures below this spatial scale.
53 Although light microscopy has been used to study living organisms since the 17th century, the
54 diffraction limit has severely restricted its ability to study subcellular organization at the scale of
55 molecules or molecular complexes. For example, the structure of a nuclear pore complex, which
56 is made up of hundreds of individual proteins, remains obscured by conventional light microscopy
57 because the entire complex has a diameter of only ~120 nm (**Fig. 1b**). Over the past two decades,
58 however, microscopy methods have emerged that overcome the diffraction limit and now enable
59 insights into biological structures such as nuclear pores, viruses, chromatin complexes or
60 cytoskeletal filaments closer to molecular scales². The most well-known of these super-resolution
61 microscopy methods fall into three main categories: stimulated emission depletion (STED)³,
62 structured illumination microscopy (SIM)^{4,5}, and single molecule localization microscopy (SMLM)⁶⁻
63 ⁹ - the focus of this Primer. SMLM usually employs conventional widefield excitation, and unlike
64 STED or SIM achieves super-resolution by localizing individual molecules⁶⁻¹⁵. Although each
65 super-resolution method has its unique advantages and limitations and is optimally suited for
66 different applications, as discussed elsewhere², SMLM has become broadly adopted in the life
67 sciences owing to its high spatial resolution and relative ease of implementation.

68 SMLM is fundamentally based on the fact that the spatial coordinates of single fluorescent
69 molecules (also called fluorophores, or emitters) can be computed with high precision if their PSFs
70 are isolated (i.e. do not overlap). This is true because, with sufficient oversampling
71 (magnification), subpixel shifts of the coordinates lead to predictable changes in pixel intensities
72 (**Fig. 1c**). Localization precision can be defined by standard deviation of the scatter of localizations
73 that would be obtained if a molecule was imaged and localized many times. For a single molecule,
74 this precision is fundamentally limited by the number of photons relative to background and noise
75 — the signal-to-noise ratio (SNR), with higher SNR allowing better precision — and not by the
76 wavelength of light or the pixel size (**Fig. 1d**). In conventional widefield microscopy, nearby
77 fluorescent molecules will have overlapping PSFs, preventing their localization (**Fig. 1b,f**). The
78 central idea underlying SMLM is to avoid these overlaps by separating the fluorescent emissions
79 of distinct molecules in time. The most common approach to obtain this temporal separation
80 employs the fact that many fluorescent molecules can switch between an 'active' (or 'bright'/'ON')
81 state, where they emit fluorescent light when excited, and one or more 'inactive' (or 'dark'/'OFF')
82 states in which they do not (**Fig. 1e**). While this photo-switching of a particular molecule is a
83 stochastic (i.e. random) event, the switching probabilities can be experimentally modulated –
84 using specific fluorophores, laser irradiation, controlling the chemical environment or other means.
85 Under suitable conditions, this allows one to ensure that at any given time only a small number of
86 molecules are ON, and hence appear as spatially isolated, non-overlapping PSFs. Many
87 thousands of image frames of the same field of view (FOV) are then typically acquired
88 sequentially, so that many (ideally all) fluorescent molecules are ON in at least one frame of the
89 sequence (**Fig. 1g**). The OFF/ON/OFF switching leads to a 'blinking' of the fluorophores in this

90 sequence of diffraction-limited images. These images are then processed computationally to
91 detect all ON molecules (**Fig. 1h**) and determine their coordinates (**Fig. 1i**). Finally, all these
92 localizations are accumulated to assemble a single image, whose resolution is no longer limited
93 by optical diffraction (**Fig. 1j-l**). The resolution of this image is determined by the accuracy and
94 precision with which individual molecules are localized, and by the distance between molecules
95 whose coordinates contribute to the image, which itself depends on the density of molecules and
96 the percentage of molecules that have been localized (**Box 1**).

97 While this general concept of sequentially imaging sparse random subsets of fluorophores
98 and computing their positions is common to most forms of SMLM, there are multiple ways to
99 perform SMLM, wherein technical choices will impact the spatial and temporal resolution, the
100 ability to perform imaging in 2D or 3D, in one or multiple colors, in fixed or live cells and more.
101 Instead of generating super-resolution images, very similar experimental approaches to SMLM
102 can be used to track large numbers of single molecules in live cells in an approach known as
103 'single particle tracking PALM' (spt-PALM)¹⁶.

104 A first key choice concerns the fluorophores and how they can be induced to switch
105 between ON and OFF states. Fluorescence photoactivated localization microscopy^{6,7,17}
106 ((F)PALM) was initially demonstrated using fluorescent proteins (FP) that can be activated by UV
107 illumination, while (direct) stochastic optical reconstruction microscopy ((d)STORM) and closely
108 related methods^{8,9,18} are based on synthetic fluorophores (dyes) that can photoswitch in the
109 presence of suitable buffers. Point Accumulation In Nanoscale Topography¹⁰ (PAINT) is a
110 different SMLM approach that does not require photoswitching, but instead relies on fluorophores
111 that switch between free diffusion and immobilization by binding to a target. The most prominent
112 variant of PAINT is DNA-PAINT¹⁹, where transient immobilization is achieved by hybridization of
113 DNA strands.

114 A second key parameter is the labeling, i.e. how fluorophores are attached to the target
115 molecule of interest, e.g. by genetic fusion with a FP or with antibodies.

116 A third important consideration for SMLM is how samples are prepared, e.g. whether and
117 how they are chemically fixed and what buffer is used to enable or promote photoswitching.

118 Fourth, while the hardware requirements of most SMLM setups are relatively basic, the
119 details of the optical system affect the resolution and ability to perform multicolor and 3D imaging.

120 A fifth choice is how image sequences are acquired, e.g. how many frames are taken and
121 with what exposure time, and how laser power is set

122 Sixth, it is crucial to use appropriate software to computationally detect and localize (and
123 possibly track) single molecules with high fidelity, and, if necessary to quantify the data.

124 Finally, SMLM can suffer from a number of artifacts such as sample drift or overlapping
125 PSFs, that need to be addressed to ensure high quality images.

126
127 This Primer article aims to provide an introduction to all main facets of SMLM and some closely
128 related approaches. The Experimentation section discusses experimental strategies including
129 choice of fluorophores, labeling, sample preparation, optical system, and image acquisition. The
130 Results section discusses how to computationally reconstruct and quantify SMLM images. The
131 Application section highlights examples of biological applications made possible by SMLM. The
132 remaining sections address issues of reproducibility and data deposition, explore limitations and

133 optimizations of current SMLM techniques, and finally provide an outlook on advanced and
134 promising new approaches.

135 [H1] Experimentation

136 SMLM techniques critically require that the molecules to be imaged (typically, but not exclusively,
137 proteins) be fluorescently labeled. In an ideal SMLM experiment, the function, location and
138 number of target molecules would be preserved by the fluorescent labeling and each target
139 molecule would be tagged at its exact position by exactly one fluorophore of minimal physical size
140 (although some advanced studies label multiple sites of the same protein or DNA molecule to
141 determine their 3D conformation, eg. ^{20,21}). To separate the fluorescent signals in time, each
142 fluorophore would ideally be briefly active in one image frame and inactive otherwise, hence have
143 high contrast ratio, and when active should emit a large number of photons relative to the
144 background to enable high SNR and localization precision. For live cell super-resolution imaging,
145 active fluorophores should also become inactive very quickly to enable rapid turnover and
146 sampling, and imaging conditions should be compatible with maintaining the cellular physiology.
147 Below, we discuss some of the main fluorophores and labeling approaches for SMLM relative to
148 these ideal requirements. **Table 1** lists a selection of recommended ingredients for newcomers to
149 SMLM.

150 [H2] Fluorophores for SMLM and how to switch them

151 SMLM fluorophores fall in one of five classes, depending on how they switch between ON and
152 OFF states (**Fig. 2a-e**). Some of these classes include both synthetic dyes and FPs. Compared
153 to FPs, synthetic dyes generally have higher photon counts and therefore better localization
154 precision. FPs are generally more suited for live cell applications, but premature bleaching and/or
155 incomplete labeling can further limit the structural resolution (**Box 1**). Note that for many
156 fluorophores, the exact molecular mechanisms underlying photoswitching are still under
157 investigation. We detail these five types of fluorophores and how to use them in SMLM below.

158

159 [H3] Photoswitchable fluorophores

160

161 Photoswitchable fluorophores (**Fig. 2a**) undergo reversible transitions from an ON to an OFF-
162 state, such that a single fluorophore can blink many times. The first synthetic dyes used for SMLM
163 were photoswitchable dyes, including carbocyanine dyes (Cy5, Alexa Fluor 647), rhodamine and
164 oxazine dyes (e.g. most Alexa Fluor and ATTO dyes)²². These dyes can be reversibly switched
165 between ON- and OFF-states upon irradiation with light of appropriate wavelength and using a
166 photoswitching buffer consisting of PBS, 10-100 mM of a thiol such as mercaptoethylamine
167 (MEA), and an enzymatic oxygen scavenger if necessary. Optimized buffers are available
168 commercially (**Table 1**). Typically, UV light ($\lambda_{ON}=405$ nm) promotes activation from OFF to ON,
169 while light of longer wavelength (e.g. $\lambda_{OFF}=650$ nm for Cy5) excites fluorescence and induces
170 transition from ON to OFF. This photoswitching behaviour occurs either in the presence²³ or

171 absence of an activator dye²⁴ and laid the foundation for STORM⁸ and *direct* STORM (*d*STORM)⁹,
172 respectively.

173
174 At the beginning of the experiment, all fluorophores reside in their ON-state. Hence, cells can be
175 easily identified and brought into focus and, standard widefield images can be recorded for later
176 comparison. To enable SMLM, the vast majority of fluorophores must then be turned OFF. This
177 usually requires high irradiation intensities ($\sim 10\text{-}30\text{ kW cm}^{-2}$ at λ_{OFF}) over a few seconds. Once
178 the density of active dyes is low enough to allow single molecule detection, the irradiation intensity
179 can be decreased to $\sim 1\text{-}3\text{ kW cm}^{-2}$. This excitation pumps the active dyes to dark states (inactive
180 triplet states), which are reduced by the thiol at pH > 7.0. The ON-state lifetime can be adjusted
181 by the irradiation intensity, the thiol concentration, and the pH of the photoswitching buffer and is
182 typically 5-20 ms, but can vary from 1 ms to 500 ms²⁵. A recent study promotes the use of low
183 excitation intensity and long exposure time to minimize initial photobleaching and improve
184 resolution²⁶. The reduced OFF-states exhibit a stability of several seconds before they are
185 transferred back to the singlet ground state upon reaction with residual oxygen²⁷. Direct excitation
186 of the OFF-state at $\lambda_{\text{ON}}=405\text{ nm}$ can be used to increase the activation and localization rate, e.g.
187 for low density target molecules. While carbocyanine dyes perform better in the absence of
188 oxygen, some oxazine dyes such as ATTO 655 also exhibit photoswitching in the presence of
189 oxygen and require generally lower thiol concentrations. Therefore, it is recommended to vary the
190 oxygen concentration to optimize the blinking performance for new dyes in (*d*)STORM
191 experiments. Since cells contain the thiol glutathione at millimolar concentrations, some synthetic
192 dyes can be used advantageously for live-cell *d*STORM²⁸. For novices, we recommend to start
193 with Alexa Fluor 647 or Cy5 in their first *d*STORM experiments (**Table 1**).

194
195 Photoswitchable fluorescent proteins (FPs) such as Dronpa²⁹ can be toggled reversibly from an
196 OFF state to an ON state upon irradiation with different excitation wavelengths ($\lambda_{\text{ON}}=405\text{ nm}$,
197 $\lambda_{\text{OFF}}=488\text{ nm}$) without requiring any special buffer. Moreover some “standard” FPs (e.g. YFP) can
198 undergo a spontaneous recovery from OFF to ON states after partial photobleaching, but their
199 use leads to localizing only a small subset of molecules, hence limiting resolution (**Box 1**).

200

201 **[H3] Photoactivatable fluorophores**

202

203 Photoactivatable fluorophores (**Fig. 2b**) essentially reside in their OFF state at the beginning of
204 the experiment, i.e. the sample is dark, but these fluorophores can irreversibly switch to an ON
205 state, either spontaneously or upon UV-light ($\lambda=405\text{ nm}$) activation under aqueous conditions.

206 Synthetic photoactivatable dyes include photochromic rhodamine amides³⁰, the azetidinyI Janelia
207 Farm (JF) rhodamine PA-JF₅₄₉ and silicon-rhodamine PA-JF₆₄₆³¹, and the bridged carbocyanine
208 Cy5B³². Because fluorophores are initially OFF, activation with 405 nm light is usually required to
209 focus the microscope. To allow single molecule localization, photoactivated fluorophores must be
210 photobleached, i.e. excited long enough until they permanently turn OFF, prior to activation of
211 new fluorophores The PA-JF dyes exhibit usually longer ON-state lifetimes and deliver higher
212 photon yields and localization precisions than the more common dyes they are derived from (e.g.
213 $\sim 5\text{ nm}$) but, on the other hand, require longer total acquisitions times, typically exceeding several

214 hours. A benefit of these dyes is their membrane permeability, rendering them ideally suited for
215 live-cell SMLM.

216

217 Photoactivatable fluorescent proteins include PA-mCherry, PA-mRFP ($\lambda_{ON}=405$ nm, $\lambda_{exc}=564$
218 nm), PA-mKate ($\lambda_{ON}=405$ nm, $\lambda_{exc}=586$ nm) and PA-GFP³³ ($\lambda_{ON}=405$ nm, $\lambda_{exc}=488$ nm) and
219 have been used in the first demonstration of (F)PALM in fixed cells^{6,7} as well as in live cells^{16,34}.
220 For most live-cell applications, photoactivatable FPs such as PAmCherry or PAtagRFP, are
221 doubtless labels of choice. However, compared to most synthetic dyes, FPs have only moderate
222 photostability and photon counts, reducing the localization precision³⁵ and requiring long
223 acquisition times.

224

225 [H3] Photoconvertible fluorophores

226

227 Photoconvertible fluorescent proteins (**Fig. 2c**) including Eos³⁶ (and its derivatives like mEos2),
228 Dendra2³⁶ and mMaple³⁷ can switch irreversibly from one spectral state (i.e. color) to another
229 upon irradiation with light of appropriate wavelength. For example, Eos can be switched from
230 green to red, by irradiation at $\lambda_{G\rightarrow R}=405$ nm, and is excited at $\lambda_G=488$ nm and $\lambda_R=561$ nm,
231 respectively. For Dendra2, $\lambda_{G\rightarrow R}=405$ nm, $\lambda_G=488$ nm and $\lambda_R=520$ nm.

232

233 The signal of the (usually weaker) shorter wavelength fluorescence (before photoconversion) can
234 be used to focus the sample and obtain a diffraction limited image before starting the SMLM image
235 acquisition sequence. As for the photoactivatable fluorophores, after photoconversion, activated
236 fluorophores must be photobleached to avoid PSF overlap with newly converted fluorophores,
237 and the photobleaching time can be controlled by the irradiation intensity. Photoconvertible FPs
238 are good choices for live cell SMLM.

239

240 [H3] Spontaneously-blinking dyes

241

242 Spontaneously blinking dyes (**Fig. 2d**) are based on a pH-dependent chemical reaction
243 (intramolecular spirocyclization) and enable SMLM at defined pH in an aqueous solution³⁸ without
244 requiring a photoswitching buffer. These dyes include the silicon-rhodamine dye HMSiR³⁹,
245 HEtetTFER⁴⁰, and FRD⁴¹. These fluorophores exhibit excellent photon yield, are partially
246 membrane permeable, and can thus be used advantageously in live-cell SMLM experiments.
247 Spontaneously blinking fluorophores show immediate blinking from the onset of an SMLM
248 experiment. The blinking kinetics is independent of the irradiation intensity and mainly controlled
249 by the pH of the aqueous buffer. ON-state lifetimes vary in the range of 10-300 ms³⁸.

250

251 [H3] Temporarily binding dyes and DNA-PAINT

252

253 Unlike (F)PALM and (d)STORM, PAINT does not require fluorophores to switch between OFF
254 and ON states. Instead, PAINT uses dyes¹⁰ or dye-labeled ligands⁴² that freely diffuse until they
255 interact with targets of interest by either permanent or transient binding (**Fig. 2e**). Because free

256 dyes diffuse rapidly over many pixels during acquisition of a single image frame, they only appear
257 as a blurred background and are not localized, whereas bound dyes appear as a PSF and are
258 localized. This strategy effectively decouples the switching between ON and OFF-states from the
259 dye photophysics. PAINT is not prone to photobleaching since the fluorophore reservoir can be
260 replenished, and bright and photostable fluorescent dyes can be used. Any synthetic dye that
261 exhibits a sufficient fluorescence quantum yield is compatible with PAINT.

262 DNA-PAINT⁴³ is a specific variation of PAINT that uses short (typically 6–10 nucleotide long) dye-
263 labeled DNA oligonucleotides (called ‘imager strands’) that can transiently, yet sequence-
264 specifically interact with their target-bound complements (called ‘docking strands’) (**Fig. 2e**). In
265 DNA-PAINT, the ON-times (also called bright times, τ_b) can be almost arbitrarily tuned by the
266 stability of the DNA duplex, as they are directly linked to the dissociation rate k_{OFF} of the imager-
267 docking-duplex via $\tau_b = 1/k_{\text{OFF}}$. Typical τ_b values are approximately 500 ms for an 8 bp duplex
268 consisting of two CG and 6 AT pairs. As a rule of thumb, increasing the length by one bp results
269 in roughly an order of magnitude increase in τ_b ⁴³. Fluorescence OFF-times (also called dark times,
270 τ_d) are linked to DNA hybridization rates (k_{ON}) and imager concentration c_i via $\tau_d = 1/(c_i \cdot k_{\text{ON}})$
271 and can be tuned by either changing c_i or k_{ON} . Bright times can be tuned to extract the highest
272 number of photons per single binding event, resulting in high localization precisions down to ~1
273 nm^{19,44}.

274

275 [H2] Fluorescent labeling strategies

276 Several options are available to permanently or transiently link SMLM-compatible fluorophores to
277 the molecules of interest. The main options are genetic fusion of a FP to the target protein;
278 immunolabeling with synthetic dye conjugated antibodies; genetic fusion of the target protein with
279 a tag that can bind to synthetic dyes; direct binding of synthetic dyes for specific structures and
280 using labels for PAINT and DNA-PAINT. The choice of labeling strategy is critical, as it contributes
281 to determining the resolution via the linkage error, i.e. the distance between the target molecule
282 and the fluorophore (**Fig. 2f**), and via the labeling density. It also determines whether the target
283 molecule retains its physiological localization and whether live cell imaging is possible.

284

285 [H3] Encoding fluorescent proteins

286 Genetically-encoded fluorescent proteins (FPs) are the most prominent labels for live cell
287 fluorescence microscopy and have been used early on for (F)PALM in fixed⁶ and live cells^{16,45}.
288 One labeling approach is to transfect cells using an expression vector containing DNA constructs
289 consisting of the gene encoding the target protein genetically fused to the desired FP⁴⁶ (**Table 1**).
290 An important disadvantage of transient transfection is that the number of expressed target
291 proteins can vary widely. In stably transfected cell lines, the protein gene is overexpressed, to
292 levels that can be controlled by construct optimization, but the endogenous protein is left
293 unlabelled. By contrast, CRISPR knock-ins allow for homozygous labeling of the endogenous
294 genes, such that protein abundance is not altered and all target proteins are labeled. FPs exhibit
295 a size of 2-5 nm and can thus also perturb protein functionality⁴⁷. Nevertheless, in the ideal case

296 each protein of interest carries a single FP and shows wild-type functionality. For more details,
297 see ref.^{33,45,48,49}.

298

299 **[H3] Immunolabeling**

300 Since synthetic dyes cannot be genetically encoded into biomolecules, they generally need to be
301 coupled to other compounds, which can bind to a target molecule. Classical immunostaining, in
302 which an antibody directed against an antigen enables binding of a specific biomolecule of
303 interest, remains the standard method for labeling endogenous proteins in fixed cells. In direct
304 immunolabeling, the antibody is chemically coupled to a synthetic dye (e.g. via NHS-chemistry),
305 but indirect labeling using a second antibody that targets the primary antibody is often used to
306 ensure higher and sensitivity and specificity (**Table 1**). Indeed, the binding of multiple secondary
307 antibodies to a single primary antibody amplifies the fluorescent signal, and unlabeled primary
308 antibodies can have better epitope binding affinity than primary antibodies modified for fluorescent
309 labeling and hence less background. An important drawback of immunolabeling in SMLM,
310 however, is that the commonly used immunoglobulin G antibodies are large (~10 nm), which
311 entails a significant linkage error, especially with indirect labeling (linkage errors of ~20 nm).
312 Camelid antibodies (nanobodies) are substantially smaller (~2.5-4 nm) and therefore offer an
313 attractive alternative, especially for proteins tagged with GFP, for which highly specific
314 nanobodies are available⁵⁰, or in combination with primary antibodies.

315 Since immunostaining of intracellular proteins requires permeabilization of cell membranes, it is
316 also generally incompatible with live cell imaging, except for extracellular or membrane proteins,
317 and in the latter case can modulate biological functions. The recent development of cell
318 permeable nanobodies may however facilitate live cell immunolabeling in the future⁵¹, as do
319 electroporation based methods⁵². In general, it is important to check that the immunolabeling,
320 permeabilization and fixation conditions allow specific labeling of the target proteins without
321 artifacts⁵³.

322

323 **[H3] Protein tags**

324 To ensure specific labeling with minimal linkage error, an alternative are biorthogonal peptide-
325 motifs or self-labeling protein tags including FIAsH tags, lipoic acid ligase, SNAP-tags, and Halo-
326 tags⁵⁴⁻⁵⁶. These tags can be genetically co-expressed with a target protein and covalently bind
327 their respective reagent or ligand, which is directly coupled to a fluorophore. These labeling
328 methods combine genetic expression with the excellent photophysical properties of synthetic
329 dyes, while reducing the linkage error to a few nanometers. Furthermore, they can be used for
330 live-cell imaging experiments as long as the dye-substrate exhibits cell membrane permeability⁵⁷.

331

332 **[H3] Direct labeling**

333 Some biological structures can be labeled using dye conjugated small peptides or drugs, such as
334 the bicyclic heptapeptide phalloidin or the taxane paclitaxel, which target actin and microtubule
335 filaments, respectively^{58,59}. However, despite their small size and minimal linkage error, such
336 labels often impair biophysical function and act as biological modulators. Additionally, some

337 modified fluorophores can be directly integrated into the biostructure itself (e.g. organelle trackers
338 or modified lipids)⁶⁰.

339 Regarding polypeptides or proteins, the smallest linkage error can be achieved via site-specific
340 labeling of a single amino acid. Finally, genetic code expansion enables incorporation of custom-
341 designed unnatural amino acids, such as TCO*-lysine, which can be directly coupled to
342 functionalized synthetic dyes via fluorogenic click reactions without affecting biological
343 functionality⁶¹⁻⁶³ and enabling live cell imaging.

344

345 **[H3] PAINT and DNA-PAINT labeling**

346 PAINT imaging has initially been restricted to imaging organelles for which transiently binding
347 fluorescent molecules exist^{10,42,64}. For example, PAINT was originally demonstrated by imaging
348 large unicellular vesicles with the fluorophore Nile Red, which is only weakly fluorescent in water
349 but becomes much brighter in a lipid environment¹⁰ and has been applied to imaging DNA with
350 dyes such as Hoechst⁶⁵. DNA-PAINT, by contrast, can be used for any target molecule that can
351 be linked to a docking strand. For this purpose, the most common approach is again
352 immunolabeling, with unlabeled primary antibodies targeting the protein of interest and secondary
353 antibodies conjugated to DNA docking strands using e.g. standard maleimide chemistry^{19,66}. To
354 overcome the large linkage errors of antibodies, DNA-PAINT has recently been combined with
355 smaller and more efficient labeling reagents including nanobodies⁶⁶⁻⁶⁸, genetically-encoded
356 tags⁶⁷, affimers⁶⁹ and novel aptamer probes called SOMAmers⁷⁰.

357 **[H2] Sample preparation**

358 To achieve the sampling required for high resolution, dense structures such as the actin
359 cytoskeleton generally require long acquisition times exceeding minutes. Therefore, cells and
360 tissue samples must generally be fixed. Nevertheless, sparse and/or slowly moving cellular
361 structures, such as focal adhesions³⁴ can to some extent be investigated using live cell SMLM.
362 Below, we discuss sample preparation for fixed samples and live cell imaging.

363

364 **[H3] Fixed samples**

365

366 SMLM in fixed samples demands chemical fixation methods that crosslink proteins, preserve their
367 binding sites and do not destroy molecular interactions. Aldehyde-based fixatives such as
368 paraformaldehyde (PFA) and glutaraldehyde have been widely used in fluorescence imaging
369 applications and are still among the most common fixation reagents for SMLM. Alternatives
370 consisting of alcohols (e.g. ice-cold methanol) show efficient fixation especially for cytoskeletal
371 components, but are unsuited to preserve the ultrastructure and molecular organization of cellular
372 membranes or organelles. In addition, glyoxal has been suggested as an alternative fixative with
373 improved preservation of cellular morphology for super-resolution microscopy⁷¹. For the
374 investigation of dynamic biological processes or protein-protein interactions in membranes,
375 residual mobility of membrane components must be considered, since it can induce artifacts such

376 as antibody cluster formation. To avoid residual mobility, membrane components should always
377 be fixed using 4% PFA and 0.2% glutaraldehyde for 30 min or longer^{72,73}.

378 For immunolabeling, cells must also be permeabilized. After fixation and permeabilization, a
379 blocking buffer such as bovine serum albumine (BSA) in PBS or normal goat serum (NGS) can
380 be used to reduce non-specific binding and background signal before labeling²⁷. In cryofixation,
381 samples are very rapidly cooled down to cryogenic temperatures (<-150°C) under high pressure
382 to prevent the formation of ice crystals. Although cryofixation is technically more demanding, has
383 been mostly restricted to thin sections⁶ and can affect photoswitching properties, it is compatible
384 with both SMLM and electron microscopy and achieves optimal preservation of cellular
385 ultrastructure^{74,75} (see Outlook).

386

387 **[H3] Live cells**

388

389 For live cell experiments, after expression of the protein of interest and FP or labeling with tag
390 substrates, cells are imaged in phenol red free medium or PBS. A major limitation of live cell
391 SMLM are light-induced defects caused by high laser intensities (phototoxicity), especially at
392 shorter wavelengths. A useful test for phototoxicity is to grow cells in medium overnight at 37°C
393 and 5% CO₂ and check their survival using cell proliferation assays. A much more sensitive
394 viability parameter is, however, if the irradiated cells undergo cell division during the next 20-24
395 hours after the SMLM experiment⁷⁶. The illumination mode, irradiation wavelength and sample
396 temperature should be adjusted to minimize phototoxic effects⁷⁶.

397

398

399 **[H2] Optics of SMLM**

400 Unlike other super-resolution methods, SMLM requires only a wide-field microscope equipped
401 with standard continuous wave lasers for excitation and activation and a sensitive camera to
402 detect single molecules (**Fig. 3a,b**). As a result of this relative simplicity, numerous commercial,
403 low-cost open hardware, and homebuilt implementations exist⁷⁷⁻⁷⁹. SMLM compatible cameras
404 are either electron-multiplying charge-coupled devices (EM-CCDs) or (back-illuminated) scientific
405 complementary metal-oxide semiconductor (sCMOS) cameras. EM-CCDs use electron
406 amplification gains that allow detection of single photons with negligible read-out noise and are
407 particularly suited for low photon counts, despite multiplicative noise due to the stochastic
408 amplification process. Modern sCMOS cameras have inhomogeneous read-out noise that
409 requires extra correction, are somewhat less sensitive for weak signals, but have higher frame
410 rates, larger chips (enabling larger FOV) and achieve similar SNR for bright dyes. The microscope
411 body itself can be a standard commercial one, but many custom microscopes use more
412 economical, minimal bodies and may lack an eyepiece and brightfield illumination (**Fig. 3b**).
413 Because microscopes require much higher stability for SMLM than conventional microscopy,
414 most commercial or custom microscopes include automated systems to keep the specimen in
415 focus. For multicolor microscopy (see also **Box 2**), multiple laser lines can be combined and co-
416 aligned using dichroic mirrors, which reflect and transmit only select wavelengths of light. Several

417 simple approaches are available to enable SMLM in 3D, e.g. through PSF engineering by
418 inserting a cylindrical lens into the optical path⁸⁰ (**Fig. 3d,e, Box 4**). Widefield illumination of the
419 sample can be generated by expanding a laser beam's circular intensity profile, then cropping the
420 FOV to keep only the relatively uniform central area.

421 The sample's fluorescence emission is imaged through an objective lens onto the detector.
422 Typically, a 60-100x, high numerical aperture (NA = 1.4 or higher) oil immersion objective is used
423 to ensure efficient photon collection. When imaging samples close to the coverslip such as cellular
424 membranes, total internal reflection fluorescent (TIREF)⁸¹ or highly inclined and laminated optical
425 sheet (HILO) illumination can be used to diminish out-of-focus background and thereby improve
426 SNR and localization precision⁸². A dichroic is again used to separate excitation and emission
427 light; for multicolor imaging a dichroic with multiple bandpasses can be chosen to reflect the
428 excitation laser lines and pass emission wavelengths. For detection, the camera pixel size should
429 be well-matched to the PSF size. The optimal magnification can be determined theoretically for
430 specific background and photon numbers^{83,84}, but as a rule of thumb the pixel size should be
431 approximately equal to half the PSF width. For example, for Alexa 647 dyes imaged with NA=1.4,
432 the PSF width (FWHM) is 290 nm. With a 60x objective and an EM-CCD camera with 16 μm
433 pixels, the pixel size in the sample plane is 267 nm, and hence too large, while with a 100x
434 objective, the pixel size is diminished to 160 nm, allowing better localization precision.

435

436 **[H2] Data acquisition in SMLM**

437 In SMLM, the number of active fluorophores in each frame should be low enough to avoid PSF
438 overlaps, but should be as high as possible to minimize acquisition time. However, experimentally
439 optimizing active fluorophore density can be challenging; for photoswitching dyes, it results from
440 a balance between excitation and activation laser intensities, and for PAINT from binding affinities
441 and binder concentration. While these parameters are usually determined manually, SMLM can
442 be automated with control software that implements a feedback loop to tune active fluorophore
443 density⁸⁵⁻⁸⁷.

444 **[H3] Structural imaging in fixed cells**

445 High-resolution studies of biological structures are generally done on fixed or purified samples,
446 which allow for long acquisitions, to maximize the number of fluorophores localized and minimize
447 motion artifacts.

448 The exposure time of each frame should ideally match the single molecule ON state lifetime,
449 which is typically ~10-100 ms (see above). The total number of frames needed to reconstruct an
450 image depends strongly on the structure being imaged and the desired resolution (**Box 1**). For
451 higher-dimension structures (e.g. 2D membranes vs 1D filaments), the signal from an ON
452 fluorophore will overlap with a larger portion of the structure and thus require more frames to
453 reconstruct⁸⁸. An accurate assessment requires a detailed analysis^{34,88,89}, but simple rules of

454 thumb give a rough estimate. For example, a structure like the centriole can be approximated as
455 a hollow cylinder 250 nm in diameter and 400 nm long, for an area of $3 \times 10^5 \text{ nm}^2$, fitting into a
456 single PSF. To resolve its surface at a scale of 20 nm would require that neighboring localizations
457 should be at least that close to each other, but due to stochasticity and the need to oversample
458 to build up the image, it ends up being at least 10 times as many, so approximately 5-10,000
459 molecules. With only one molecule 'ON' per frame, this implies that at least 10-20,000 frames
460 should be collected. With a typical per-frame exposure time of 10-100 ms, this implies an
461 acquisition time of 2-30 minutes.

462 **[H3] Structural dynamics in live cell SMLM**

463 For SMLM studies in live cells, two main imaging modes can be distinguished: structural dynamics
464 and molecular dynamics (**Fig. 4**). Structural dynamics aims to reveal the time evolution of a
465 structure composed of many molecules, such as a focal adhesion, a clathrin coated pit, or an
466 organelle membrane^{34,60,90}. In these studies, time series of super-resolution images are
467 constructed from the localizations obtained in consecutive (non-overlapping) sets of frames (**Fig.**
468 **4a**). Choosing the number of frames in each set faces two conflicting requirements: more frames
469 imply diminished temporal resolution and potentially motion blur, while less frames imply sparse
470 sampling of the structure and hence worse spatial resolution³⁴ (**Box 1**).

471 **[H3] Single molecule dynamics in live cells (spt-PALM)**

472 The goal of molecular dynamics is to follow the trajectories of single molecules, often considering
473 them as a probe of local environmental properties (**Fig. 4b**). Individual molecules are photo-
474 converted, identified and tracked over multiple frames, in an approach first introduced as 'spt-
475 PALM'¹⁶. In order to avoid misconnecting trajectories, the distance between molecules in each
476 frame should be several times larger than the distance they travel between consecutive frames
477 ^{16,42}. This implies that the density of active fluorophores must be significantly lower than for
478 structural dynamics. Fluorophore motion between adjacent frames becomes a desired
479 characteristic rather than a problem, so the time between frames should be chosen to allow
480 molecules to move a distance greater than their localization precision. Although single molecule
481 tracking existed before SMLM^{91,92}, the repeated photoactivation or photoconversion of
482 fluorophores in live cells allows collection of orders of magnitude more molecular trajectories and
483 hence enables much more detailed insights into molecular movements and the factors that control
484 them⁹³.

485 **[H1] Results**

486 In SMLM, super-resolution images are not seen through the eyepiece of the microscope, but are
487 assembled in a computer. Thus, the quality of super-resolution images strongly depends on image
488 processing. Below, we discuss the computational aspects of SMLM data analysis. The majority
489 of SMLM reconstruction methods can be decomposed in three main consecutive steps: single

490 molecule detection, single molecule localization, and super-resolution image rendering. We
491 discuss these three steps in more detail below.

492 [H2] Single molecule detection

493 In the detection step, each diffraction-limited image is processed to determine the approximate
494 location of active emitters. Before this, the image is often preprocessed to remove
495 inhomogeneous background, e.g. using rolling ball, difference of Gaussians or wavelet filtering⁹⁴
496 or by estimating the background from the entire image sequence⁹⁵. Detection is then typically
497 done by extracting local maxima from the background-corrected image. Alternatively, the raw
498 image can be searched for PSF-like patterns, e.g. by computing the correlation of this image with
499 a small image representing the model PSF (e.g. a Gaussian approximation of the Airy function,
500 or a more elaborate model for engineered PSFs in 3D SMLM, see **Box 4**) and thresholding the
501 correlation image. The result is usually a set of pixel regions likely to contain single molecules. It
502 can be shown mathematically that any detection algorithm will make false positives and/or false
503 negatives, whenever images are corrupted by noise⁹⁶. The lower the SNR of the image, the more
504 unavoidable errors. Cameras used in SMLM can in principle have several sources of noise,
505 including additive noise from thermal electrons (dark current noise), read-out noise, or
506 multiplicative noise from stochastic amplification of photoelectrons. While read-out is usually
507 negligible, dark noise can be reduced by using cooled cameras and multiplicative noise by setting
508 low amplification gain, a fundamentally inevitable noise comes from the fact that fluorophores and
509 the background emit only a finite number of photons, leading to Poisson noise. High photon counts
510 relative to the background imply high SNR and therefore better detection performance.

511 [H2] Single molecule localization

512 In the crucial localization step, the pixel regions determined above are analyzed further to
513 compute the subpixelic (x, y) coordinates of each molecule. We now briefly discuss the
514 fundamental limits to localization precision and how localizations are computed.

515 [H3] Fundamental precision limits

516 Because of noise, localizations cannot be computed exactly, thus algorithms will make errors.
517 These errors generally have random and systematic parts, as measured by the variance and the
518 bias^{83,84}, and by precision and accuracy, respectively (see **Box 3**). Statistical estimation theory
519 shows that the precision of an algorithm without bias is limited by the Cramer-Rao lower bound
520 (CRLB)⁹⁷. This limit depends on the SNR, with higher SNR allowing better precision. A well-known
521 form of the CRLB is: $\sigma_{loc} \geq \sigma_0 / \sqrt{N}$, where σ_{loc} is the precision, defined as the standard deviation
522 of errors in estimating coordinates, σ_0 is the standard deviation of the PSF and N is the number
523 of photons collected by the camera (see **Box 3**). Typical values are $\sigma_0 \approx 100$ nm and $N = 10^2 -$
524 10^4 , which would predict precision limits of $\sigma_{loc} \geq 1-10$ nm. However, this simple formula is
525 generally too optimistic, because it ignores factors such as non-Gaussian PSF shape, read and
526 amplification noise, background signal, finite pixel size, and dipole orientation, all of which worsen

527 the precision limit. More complex expressions have been derived that take some or all of these
528 factors into account^{84,98}, for example:

$$529 \quad \sigma_{loc} \geq \sqrt{\left(\frac{\sigma_0^2 + a^2/12}{N}\right)\left(\frac{16}{9} + \frac{8\pi\sigma_0^2 b^2}{a^2 N^2}\right)}$$

530 where a is the pixel size, and b the background intensity⁹⁸. The CRLB has become an essential
531 notion in SMLM, because it defines a fundamental limit that all localization algorithms can (and
532 should) be compared to.

533 [H3] Localization algorithms

534 Among the many different localization algorithms that have been proposed, the gold standard is
535 maximum likelihood estimation (MLE), which approaches the CRLB for high SNR^{84,98-100}. MLE
536 aims to compute the (x, y) coordinates for which the probability $p(I; x, y)$ of obtaining the actually
537 observed image I (the likelihood) is highest. To find this maximum, MLE algorithms employ an
538 iterative procedure called ‘gradient ascent’, which starts from an initial position (x_0, y_0) (e.g. the
539 center of a pixel identified in the detection step) and displaces it by small increments $(x_{n+1} = x_n +$
540 $\delta x_n; y_{n+1} = y_n + \delta y_n)$ designed to increase the likelihood, until it cannot be increased further. The
541 same approach can be extended to compute 3D (x, y, z) coordinates (**Box 4**). In practice, MLE
542 algorithms differ depending on the assumed mathematical model of image formation, including
543 assumptions on PSF shape, background and noise, and MLE implementations may differ in
544 technicalities of the gradient ascent procedure. On simulated images, MLE algorithms typically
545 reach the CRLB, suggesting that their precision cannot be improved upon. A critical requirement
546 to achieving this in practice is to have an accurate model of the PSF. The widely used Gaussian
547 models are inaccurate, however, since they do not match the Airy patterns predicted from
548 diffraction theory. More complex functions based on optical theory provide better PSF models,
549 but may depend on unknown parameters and fail to capture aberrations due to imperfections of
550 the optical system and/or induced by the sample. Therefore, it is preferable to calibrate PSF
551 models, e.g. on real images of subdiffraction fluorescent beads by cubic spline fitting or phase
552 retrieval, especially in 3D SMLM^{101,102}. Unsurprisingly, algorithms that use experimentally
553 measured PSFs outperform those based on idealized PSFs⁹⁹. Equally important is a good
554 modeling (or subtraction) of the background, which is often assumed flat, but can also lead to
555 biases in localizations if not properly accounted for. The iterative nature of MLE generally makes
556 these algorithms slower than less precise non-iterative algorithms⁷⁹. However thanks to GPUs
557 and other optimizations, state-of-the-art MLE algorithms now typically allow real-time analysis of
558 SMLM image sequences⁹⁹.

559 [H2] Postprocessing of localizations

560 After localizations are computed for all detected molecule, software apply post-processing steps
561 to filter out suboptimal localizations, e.g. those with poor computed CRLB, or whose image does
562 not match the model PSF well. Stringent filtering can easily improve the average localization
563 precision, but will not necessarily improve resolution, because rejecting localizations
564 compromises sampling (see **Box 1**). Another important post-processing step is to merge nearby

565 localizations in consecutive frames, which most likely arise from the same molecule, resulting in
566 a single, more precise localization, without degrading sampling density. In addition, most SMLM
567 experiments require drift correction, to be discussed later (see 'Limitations and optimizations').

568 [H2] Super-resolution image rendering

569 Once all diffraction-limited images have been processed, the extracted localizations are rendered
570 in a new 'super-resolution' image. Typically, a grid is defined, with pixel sizes similar to the
571 precision (say, $\sigma_{loc} = 10$ nm) and counting how many localizations fall into each bin (**Fig. 1k**). This
572 2D histogram is then visualized like any other image, with counts treated as intensities (**Fig. 1l**).
573 Minor variations of this image rendering exist. For example, more weight can be given to
574 localizations with higher intensities, or localizations can be replaced by Gaussian spots with a
575 standard deviation given by their estimated precision (and normalized to an integral of 1 as
576 probability densities). The resulting image should have sub-diffraction resolution, provided that
577 certain conditions are met, as discussed in **Box 1**.

578 [H2] Software

579 Because computational processing is crucial to SMLM, over a hundred independent software
580 packages have now been developed. To help users choose among this plethora of tools, a
581 challenge has been organized that objectively compares many localization software on simulated
582 images and a small set of experimental data, and its results are updated on a dedicated
583 website^{99,103}. Although these challenges are not without caveats (software are typically run by
584 their authors and require expert fine-tuning, and results are contingent on the chosen simulation
585 assumptions and experimental data), they provide a good starting point for state-of-the-art SMLM
586 software. See **Table 2** for a selection of recommended software packages.

587 [H2] Quantitative analyses

588 Beyond generating super-resolution images for visual inspection, it is often desirable to extract
589 quantitative information, such as the number, density or spatial distribution of molecules. The
590 nature of SMLM data as molecular localizations opens distinct possibilities, but also comes with
591 challenges. We discuss selected quantitative analyses methods for SMLM below.

592 [H3] Identifying clusters and counting molecules

593 One common question in SMLM studies of protein distributions is whether these form clusters,
594 and if so of what size and stoichiometry. Analytic tools from spatial statistics such as Ripley's K
595 function can help determine if a distribution of points in an image is clustered (or on the contrary
596 exhibits spatial dispersion) and if so over which length scales. However, applying such tools to
597 SMLM data is complicated by the fact that a single molecule often gives rise to a cluster of
598 localizations owing to stochastic blinking and localization errors. Ignoring this can lead to
599 artifactual clustering^{104,105}. One approach to distinguishing apparent single molecule clusters from
600 real molecular clusters is to determine the statistical distribution of localization errors, and apply
601 pair-correlation analysis, as was demonstrated in an early study of membrane protein clusters¹⁰⁵.

602 This early method requires careful calibration, however, and although it provided average sizes
603 of clusters, it cannot identify and measure individual ones. Analyzing clusters individually requires
604 segmenting the image into groups of localizations. One proposed solution is a Bayesian method
605 that can adapt to varying localization precisions¹⁰⁶, but requires strong assumptions on cluster
606 shape. Alternative clustering methods include the density-based spatial clustering of applications
607 with noise algorithm (DBSCAN) and tessellation-based approaches^{107,108}.

608 Once localizations are segmented into clusters, a common goal is to count the underlying target
609 molecules. This is often challenging, however, because some target molecules may be localized
610 many times, either because of repeated blinking (as in (d)STORM), or because they are labeled
611 by multiple fluorophores (as in secondary immunolabeling), whereas others may not be localized
612 at all for lack of labeling (as with partial transfection labeling) or failed photoactivation during
613 acquisition time^{104,105,109}. These confounding factors can be reduced, but not entirely eliminated,
614 with endogenous homozygous labeling by photoactivatable proteins. In general, calibration
615 standards are a useful approach to account for repetitive blinking, but also failed photoactivation
616 and variations in labeling stoichiometry^{105,110-114}. Another approach to reduce counting errors is
617 kinetic modeling of the photoswitching¹¹⁵, which has been demonstrated successfully for
618 photoactivatable proteins and shown to enable correct estimates of the number of copies of a
619 molecular motor protein in bacteria¹¹⁶. However, modeling photoswitching kinetics in the presence
620 of multiple dark states can be very complex. DNA-PAINT offers a particularly promising avenue
621 for quantification thanks to well-known kinetics of binding. Its derivative qPAINT¹¹⁷ uses the fact
622 that binding frequency (assuming constant probe influx) scales linearly with the number of docking
623 strands and thus targets. If calibrated for a single site, the number of targets in an unknown region
624 can be calculated based on the observed blinking frequency, and the virtually unlimited sampling
625 of target molecules allows minimization of statistical error.

626

627 **[H3] Single particle reconstruction**

628 For molecular complexes present in large numbers of identical copies, one can gain structural
629 information at scales below SMLM resolution, by superimposing and averaging SMLM images of
630 many individual structures after translations and rotations— as commonly done in electron
631 microscopy^{118,119}. The averaging allows measurement of structural features with a precision that
632 is only limited by the number of imaged complexes, and not by single molecule localization
633 precision. This approach has allowed determination of the radial position of nucleoporin epitopes
634 within nuclear pore complexes with sub-nanometric precision¹²⁰ and has been extended to 3D
635 applications to analyze the structure of human centrioles¹¹⁹. Single particle reconstruction is thus
636 a powerful means to determine subunit architecture of large multi-protein complexes.

637 **[H3] Colocalization analyses**

638 Multicolor fluorescence microscopy is commonly used to determine if different molecules
639 associate. In conventional microscopy, colocalization analyses typically measure the extent to
640 which the diffraction limited signals from two or more channels overlap or correlate¹²¹. In multicolor

641 SMLM, strict colocalization should theoretically never occur, since distinct molecules cannot
642 occupy the same physical location, and colocalization should be replaced by analyses of
643 distances between molecules. Because of localization errors, however, individual localizations
644 are better viewed as samples of probability densities, which can overlap for different color
645 channels if molecules are closer than SMLM resolution. In principle, a simple way to assess
646 colocalization is to apply established methods for conventional microscopy¹²¹ to pairs of super-
647 resolution images, but results can be strongly affected by stochastic blinking and/or background
648 noise. Some recent methods therefore measure colocalization based on the localizations
649 themselves, e.g. by adapting tools from spatial statistics or using tessellation-based segmentation
650 of probability densities¹²²⁻¹²⁴. More work is needed to compare and improve colocalization
651 methods against solid ground truth data. Regardless of the method used, chromatic aberrations
652 can pose serious issues and must be carefully measured and corrected using, e.g. multicolor
653 beads or fluorospheres.

654 **[H3] Single molecule tracking**

655 To quantify molecular dynamics in sptPALM¹⁶, two main approaches can be distinguished:
656 Lagrangian and Eulerian. Lagrangian methods focus on characterizing the dynamics of individual
657 molecules by following them individually, for example by computing mean-squared displacements
658 and estimating diffusion coefficients or transport states for each molecule^{16,125} (**Fig. 4b**). Eulerian
659 methods instead focus on individual regions in the sample and use the dynamics of molecules
660 passing through them to infer local properties that characterize or affect molecule dynamics, e.g.
661 maps of diffusivity or energy potentials¹²⁶. For both approaches, it is important to take into account
662 the fundamental uncertainties discussed above, to avoid biases in the analysis of molecular
663 dynamics such as apparent subdiffusion or spurious energy potentials due to random localization
664 errors^{127,128}. More extensive discussions of single molecule tracking methods can be found in
665 refs^{93,129}.

666 **[H3] Other quantification methods**

667 Many more methods have been developed to analyze SMLM point clouds in specific or generic
668 contexts. These include various methods to segment structures from the background to analyze
669 their morphologies, as shown e.g. for dendritic spines in neurons¹⁰⁸, or cytoskeletal filaments, e.g.
670 to measure their orientation, lengths or curvature^{130,131}. Another example are iterative hierarchical
671 clustering methods, to classify the morphology of protein aggregates¹³². Given the variety of
672 techniques and the current lack of comparative assessments, careful validation of each
673 quantification method on simulated and experimental data remains critical. For more complete
674 discussions of SMLM data analysis, see refs^{107,133,134}.

675 **[H1] Applications**

676 Compartmentalization of cells is crucial for regulating cell function. In recent years, SMLM has
677 enabled us to visualize the spatial organization of proteins and nucleic acids within sub-cellular

678 compartments with nanoscale resolution. Below we summarize major new biology emerging from
679 these studies, focusing on specific sub-cellular compartments including the nucleus, cell
680 cytoplasm and plasma membrane.

681 [H2] Nucleus

682 Despite being a compartment of its own, the nucleus communicates with the rest of the cell
683 through the nuclear pore complexes, transport portals that allow protein and nucleic acid
684 exchange with the cytoplasm. Advances in SMLM have facilitated the study of the cell nucleus
685 and the visualization of intranuclear structures and nuclear pores.

686 [H3] Chromatin Organization

687 Recent studies have demonstrated that chromatin is not randomly distributed inside the nucleus
688 but is intricately folded and spatially organized¹³⁵. SMLM methods are playing an important role
689 in deciphering the organization of the human genome at length scales that are inaccessible to
690 standard light microscopy (20-100 nm or 10 kB-1 Mb) and that are important for regulating gene
691 function (for a recent review see ref¹³⁶). Chromatin compaction has been thought to follow a
692 hierarchical order where nucleosomes form a 10 nm fiber that resembles beads on a string, which
693 further compacts into a 30nm fiber. However, this textbook view has been challenged in recent
694 years. SMLM enabled the visualization of “nucleosome clutches” consisting of tens to hundreds
695 of nucleosomes (few Kb in genomic scale and 20-100 nm in spatial scale) along the chromatin
696 fiber in interphase nuclei of somatic cells. Similar results, in which nucleosomes pack into
697 nanodomains of varying sizes were obtained by live cell super-resolution imaging¹³⁷ and recently
698 recapitulated at specific genomic loci using mesoscale modeling¹³⁸. While clutch size may be
699 underestimated, especially in compact chromatin regions, due to imperfect labeling, these results
700 suggest that chromatin packing in the nucleus is more heterogeneous than the regular 30 nm
701 fiber (**Fig. 5a**)¹³⁹. Clutch size was cell-type specific and depended on epigenetic histone
702 modifications^{140,141}, suggesting that it can be a regulator of gene activity. While labeling of histones
703 relies on immunostaining or over-expressing a tagged histone and the labeling efficiency depends
704 on epitope accessibility to the antibody or the incorporation of tagged histones into nucleosomes,
705 DNA itself can be labeled very efficiently using modified nucleotides and click chemistry^{140,142}.
706 Dual color SMLM imaging of histones and DNA showed a high level of co-localization between
707 the two compartments as expected, suggesting that SMLM histone images are representative of
708 chromatin organization within the nucleus¹⁴⁰.

709 Similarly, SMLM imaging of DNA labeled with Oligo-Paint probes showed that distinct epigenetic
710 states follow distinct DNA packing and spatial organization in *Drosophila* cells¹⁴³. In addition to
711 the nucleosomal level, SMLM was also used to study larger chromatin domains including
712 topologically associating domains (TADs), which are Megabase regions (100-500 nm length
713 scale) of DNA determined by genome-wide chromosome conformation capture (Hi-C), within
714 which DNA sequences interact more frequently with each other than with neighboring DNA
715 sequences outside of TADs¹⁴⁴. SMLM, in combination with multiplexed DNA-FISH (fluorescence
716 in situ hybridization) methods, showed that the boundaries of TADs are more variable in single

717 cells than previously thought using population Hi-C^{20,145}, highlighting the importance of visualizing
718 these small domains at high resolution in single cells.

719 For recent reviews of high resolution imaging of chromatin see refs.^{136,146}.

720 **[H3] Transcriptional machinery and nuclear proteins**

721 Live-cell SMLM has been used to visualize the dynamics and spatial organization of the
722 transcriptional machinery, in particular RNA Polymerase II (RNA PolII)¹⁴⁷. These studies showed
723 that RNA PolII assembled into transient nuclear clusters, with lifetimes that correlated with their
724 mRNA output, suggesting that they are transcriptionally active¹⁴⁸. Follow up work showed that at
725 least some of the RNA PolII clusters were phase separated liquid droplet condensates,
726 particularly in genome regions known as super-enhancers¹⁴⁹. SMLM further showed that several
727 nuclear proteins, including architectural proteins like CCCTC-binding factor (CTCF) and the
728 Polycomb group proteins, form nano-sized clusters within the nucleus^{150,151}. Hence, clustering of
729 nuclear proteins may be a general phenomenon for both shaping genome organization and
730 regulating transcriptional output. Live cell SMLM will be important to determine if nuclear proteins
731 form phase separated clusters that cannot be resolved with conventional microscopy.

732 **[H3] Nuclear Pore Complex (NPC)**

733 NPCs are excellent reference structure for characterizing and further improving super resolution
734 microscopy modalities^{113,152,153}, and SMLM studies have helped to elucidate the structure of
735 NPCs^{120,152}. Most recently, live-cell high-speed single-molecule microscopy enabled the
736 visualization of protein domains translocating through NPC channels and gave insight into
737 transmembrane protein transport¹⁵⁴. Finally, as previously mentioned, the arrangement of
738 individual subunits within the NPC was elucidated by combining SMLM with single particle
739 averaging^{120,152} (**Fig. 5b**). This approach promises to be a powerful method for elucidating the
740 subunit architecture of large multi-protein complexes.

741 **[H2] Cytoplasmic contents**

742 **[H3] Microtubules**

743

744 Microtubules were an early target structure to visualize with SMLM and validate SMLM methods
745 due to their known shape and size (polymers with a diameter of 30 nm), as well as the abundance
746 and ease of labeling of microtubule subunits (α -tubulin and β -tubulin)^{80 155}. Besides being good
747 test structures for SMLM, microtubules also play several important cellular functions including
748 facilitating long-range transport and cell division. Combining super-resolution imaging of
749 microtubules with single particle tracking of sub-cellular compartments enabled visualization of
750 how these compartments are transported within the complex microtubule network in the crowded
751 cell cytoplasm^{156 157 158}. These studies uncovered that a special subset of microtubule tracks that
752 are detyrosinated, play a crucial role in transporting and spatially organizing lysosomal and
753 autophagosomal compartments to regulate autophagy¹⁵⁸.

754 **[H3] Mitochondria, lysosomes and the endoplasmic reticulum**

755 The photoswitching capability of common membrane probes specific to mitochondria,
756 endoplasmic reticulum (ER) or lysosomes has further enabled visualizing the dynamics of these
757 organelles with high spatial (30-60 nm) and temporal (1-2 s) resolution, revealing for example
758 fusion and fission dynamics of individual mitochondria⁶⁰. SMLM has resolved the organization of
759 mitochondrial sub-compartments including mitochondrial inner and outer membrane, cristae and
760 nucleoids¹⁵⁹. SMLM revealed that mitochondrial nucleoids are heterogeneous in terms of their
761 shape and size, are closely associated with the inner mitochondrial membrane, and that
762 mitochondrial DNA inside nucleoids is more condensed than previously thought¹⁶⁰. Low-resolution
763 images suggested that the morphology of the ER consisted of tubules and sheets. Combining
764 lattice light sheet (see 'Outlook') and PAINT, it was shown that these apparent ER-sheets are in
765 fact very dense tubular structures, changing the textbook perception of this crucial organelle¹⁶¹.

766 [H2] Plasma membrane

767 Because the plasma membrane can be imaged with TIRF⁸¹, which affords improved resolution,
768 SMLM is ideally suited to study the molecular organization of the plasma membrane with
769 fluorescently labeled antibodies, toxins or ligands. SMLM has been instrumental in revealing that
770 membrane receptors are often not uniformly distributed but sequester into small, functional nano-
771 domains too small to be resolved with conventional light microscopy. One early PALM study
772 showed for example how clusters of GPI-anchored proteins are disrupted by cholesterol
773 depletion¹⁰⁵. Recent studies used *d*STORM to investigate the effect of inflammatory activation on
774 the clustering of tumor necrosis factor receptor 1 (TNFR1)¹⁶² or the toll-like receptor TLR4¹⁶³, and
775 sensitive detection of very low levels of an antigen in myeloma cells, with implications for cancer
776 immunotherapy¹⁶⁴. Earlier SMLM studies found that T cell antigen receptor (TCR) and linker for
777 activation of T cells (Lat) are found in separate protein islands that concatenate after T-cell
778 activation¹⁶⁵. Subsequent studies revealed that TCR nano-clusters have a functional role in
779 antigen recognition and therefore signal initiation¹⁶⁶. However, this view has been recently
780 challenged with quantitative super resolution imaging methods that attributed the clustering to
781 overcounting artifacts¹⁶⁷, emphasizing the importance of careful quantitative analyses of SMLM
782 data. More applications of SMLM to plasma membrane organization and associated challenges
783 are discussed e.g. in ref. ¹⁶⁸

784 The plasma membrane interacts with the extracellular matrix (ECM) through focal adhesions,
785 which are crucial both for cell adhesion and migration. iPALM, an advanced form of SMLM (see
786 'Outlook') was used to study the 3D organization of proteins within focal adhesions¹⁶⁹. This work
787 revealed the layered organization of focal adhesions, in which integrins and actin are separated
788 by 40nm vertically (**Fig. 5d**), a region that comprises several layers; a membrane-apposed layer
789 containing integrin tails, paxillin and focal adhesion kinase, an intermediate layer with talin and
790 vinculin and an upper layer with actin regulators such as zyxin, α -actinin and vasodilator-simulated
791 phosphoprotein. More recently, using SMLM, talin — but not vinculin — was identified as the
792 primary determinant of focal adhesion organization as it diagonally spans the 40nm focal
793 adhesion core region and acts as a molecular ruler¹⁷⁰.

794 [H2] Neuronal synapses and axons

795 Neurons are the most compartmentalized cells with a highly polarized and extended morphology
796 in which the cell soma is separated from axonal, dendritic and synaptic compartments. The size
797 of the synapse is below the diffraction limit, since the distance between the pre-synaptic and post-
798 synaptic structures is ~20-30 nm. SMLM has been particularly useful in elucidating synaptic
799 organization as it can readily resolve pre-synaptic and post-synaptic markers and determine the
800 molecular architecture of proteins within synapses¹⁷¹. For example, SMLM revealed the
801 nanoscopic organization of Bruchpilot (Brp) proteins within the synaptic active zone (AZ), a
802 structure found within the presynaptic terminal in *Drosophila*¹⁷². Quantitative analysis further
803 estimated the precise stoichiometry of the Brp proteins within nanoscopic complexes, showing
804 that there are hundreds of these proteins within the AZ arranged in several heptameric clusters¹⁷².
805 Moreover, SMLM allowed the detection of AMPA and NMDA receptor nanoclusters within the
806 post-synapse, a finding with important implications for the regulation of synaptic transmission<sup>173-
807 175</sup> and a recent dSTORM study analyzed glutamate receptor clustering at presynaptic active
808 zones of the mouse cerebellum¹⁷⁶.

809 Neuronal axons are densely packed with proteins, and therefore, challenging to image with
810 conventional microscopy. One of the most striking findings enabled by SMLM was the discovery
811 of actin rings with highly regular periodicity of <200 nm, wrapped around the circumference of
812 neuronal axons (**Fig. 5c**)⁵⁸. Moreover, spectrin also demonstrated similar periodicity to actin and
813 was further shown to be interconnected with actin, resulting in the formation of a quasi-1D lattice
814 structure, also known as the membrane-associated periodic scaffold (MPS).

815 [H2] Microbes

816 In addition to helping reveal the organization of cells, SMLM is well adapted to shed light on the
817 inner structure of microbes and on how they can subvert the cellular machinery for their own
818 purposes. SMLM is especially relevant for viruses, most of which are smaller than the diffraction
819 limit, e.g. the HIV virion has a diameter of only ~100 nm. Unsurprisingly, SMLM or spt-PALM have
820 been used to revisit the internal structure of all facets of their replication cycle from cellular entry,
821 intracellular transport, replication, assembly, release and maturation for viruses such as HIV,
822 herpes simplex virus, or respiratory syncytial virus¹⁷⁷⁻¹⁸³ and SMLM approaches are also currently
823 used to study SARS-CoV-2 infected cells. While bacteria are typically somewhat larger than the
824 diffraction limit (~1 um in width and 2-3 um in length), SMLM has also proven very useful to
825 analyze their internal architecture, for example to reveal the helical organization of the cytoskeletal
826 protein FtsZ¹⁸⁴, or the organization of nucleoid associated proteins in *E coli*¹⁸⁵. SMLM has also
827 helped to clarify how bacteria interact with host cells, e.g. by visualizing septin cages entrapping
828 *Shigella* as part of a cellular defense mechanism¹⁸⁶. For more applications of super-resolution
829 imaging to bacterial pathogens, see ref. ¹⁸⁷.

830 [H1] Reproducibility and data deposition

831 SMLM involves a much larger number of experimental and analytical parameters than
832 conventional microscopy studies. Furthermore, the intrinsic stochasticity of single molecule
833 blinking makes typical SMLM images particularly noisy. These factors pose a challenge to

834 reproducibility of SMLM experiments. Therefore, it is especially important to carefully document
835 experimental protocols and computational analyses and to make data and software available. On
836 the software side, many free and open source software tools for SMLM are now available and
837 have been compared using shared benchmarking data⁹⁹, but there is still a need to record and
838 share the analysis parameters used, such as filtering on photon counts or localization precision.
839 Competitions that benchmark downstream analyses such as cluster identification or single particle
840 reconstruction will be useful to complement the challenges on localization. Platforms that integrate
841 and compare existing tools would be useful to increase reproducibility of quantitative analyses.

842
843 On the data side, image deposition is increasingly recognized as important, leading to data
844 sharing initiatives in the microscopy community, such as through archiving on Zenodo, FigShare,
845 BioStudies and to some extent the Image Data Resource¹⁸⁸. However, the large image volumes
846 generated by SMLM experiments are an impediment to raw data sharing. Therefore, we propose
847 that a good first step is to share localization data together with minimal information about the
848 experiment. These data should include the target molecule(s) and/or epitope(s); the fluorescent
849 labeling technique used with details of antibody concentrations and the functionality of FP tagging;
850 the fixation protocol if relevant; microscope parameters such as laser wavelength, laser power
851 density, camera type, exposure time, pixel size, objective magnification, numerical aperture and
852 immersion medium; reconstruction software and parameters used, and a localization table
853 including at least x,y coordinates and frame numbers, and preferably photon counts. A community
854 resource that allows users to catalogue data doi with this information appended could catalyze
855 data sharing and reuse and improve reproducibility in SMLM.

856 **[H1] Limitations and optimizations**

857
858 Although powerful, current SMLM techniques face a number of limitations. These include
859 susceptibility to reconstruction artifacts, difficulties in imaging thick samples or tissues, and very
860 low throughput, and limited applicability in live cell imaging. In this section, we highlight some of
861 these limitations as well as optimizations to alleviate them.

862

863 **[H2] Reconstruction artifacts and solutions**

864 Several types of artifacts can affect the quality of reconstructed super-resolution images, including
865 drift, overlapping PSFs, and localization biases.

866 **[H3] Sample drift**

867 A very common issue in SMLM is sample drift relative to the objective during image sequence
868 acquisition, which is often unavoidable, even when using autofocus systems designed to limit
869 axial drift . If ignored, even small drifts can result in blurring of the reconstructed image or even
870 generate artefactual features, e.g. shadow microtubules⁷⁹ (**Fig. 6a**). However, drift can be
871 measured, typically by tracking fiducial markers, such as fluorescent beads or gold coordinates

872 (which ideally remain fluorescent for the entire experiment), and the coordinates can then be
873 corrected, simply by subtracting the estimated drift (**Fig. 6a, Table 1**). An interesting recent
874 alternative is to estimate the drift from speckle patterns generated by back-scattered light¹⁸⁹. For
875 highly redundant structures such as microtubules, the lateral drift can also be estimated directly
876 from the single molecule localizations alone using e.g. image cross-correlation, although this
877 requires more caution^{190,191}. Since axial drift can also deteriorate 2D SMLM images, 3D drift
878 correction methods should be used whenever possible^{102,189,192}. Many SMLM software packages
879 provide drift correction tools (**Table 2**). Unlike drift, high frequency vibrations that affect
880 localization precision by blurring individual diffraction-limited frames cannot be corrected
881 computationally. Therefore, it is important to isolate the microscope from sources of vibration. The
882 best way to avoid residual vibrations and drift artefacts is to eliminate them using active, real-time
883 adjustment of the stage position^{191,193,194}. For example, fiducial markers added to the coverslip
884 can be tracked with a second camera and a closed loop system to drive piezoelectrical actuators
885 that precisely reposition the stage¹⁹⁵. Such approaches, although technically more demanding,
886 can reduce drift to single nanometers or less¹⁹⁵⁻¹⁹⁷.

887 **[H3] Overlapping PSFs**

888 Although the basic principle of SMLM assumes spatially isolated PSFs, avoiding PSF overlaps
889 entirely is difficult, especially in high density regions. Furthermore, it can be desirable to increase
890 the activation probability (hence leading to PSF overlaps) to increase imaging speed. Standard
891 localization algorithms tend to treat overlapping PSFs as a single molecule and compute an
892 incorrect position located somewhere in between. This can for example lead to an artificial blurring
893 at intersecting filaments (**Fig. 6b**). In principle, such artifacts can be alleviated by stringent filtering
894 of localizations for which the image deviates from that of a single PSF. However, stringent filtering
895 can lead to reject most localizations and lead to the paradox that high density regions appear
896 dimmer than low density regions in the reconstructed image (**Fig. 6c**). A simple but effective
897 approach to detecting such artifacts is to verify if a blurred version of the super-resolution image
898 is consistent with the widefield image. This approach yields a map that can highlight regions
899 affected by reconstruction errors¹⁹⁸.

900 To avoid these artifacts and analyze images with high activation density, several ‘multi-emitter
901 fitting’ algorithms have been developed that explicitly allow for PSF overlaps^{99,103} or compute
902 differences between consecutive images to create images closer to single molecule conditions¹⁹⁹.
903 Some related methods even dispense of the notion of localizing single molecules altogether²⁰⁰.
904 These algorithms can indeed produce super-resolution images. However, overlapping PSFs
905 necessarily imply significantly poorer resolution compared to low density SMLM regimes^{99,103,201}.

906 **[H3] Other biases**

907 Several other artifacts can occur in absence of drift or PSF overlaps. One example is a localization
908 bias within the camera pixel, which can arise, for example, from ill convergence of MLE algorithms
909 due to a mismatch between the model PSF and the actual PSF, and result in images with apparent
910 grid patterns²⁰² (**Fig. 6d**). This type of bias can be easily identified from the histogram of
911 localizations relative to pixel centers (**Fig. 6d**). It is less easy to identify spatially varying biases,

912 caused e.g. by a non-uniform background. One solution here is to take into account or eliminate
913 non-uniform background by estimating it from individual images or from the entire image
914 sequence⁹⁵. Another common issue in 3D SMLM with engineered PSFs are aberrations due to
915 refractive index mismatch between sample and oil, which can cause distortions along the axial
916 dimension. Such distortions can be detected and prevented by careful calibration, typically by
917 imaging fluorescent markers at known axial positions in the sample and adjusting the PSF model
918 to fit them (**Box 4**)^{102,203,204}.

919 Another, much more fundamental and often overlooked localization bias stems from the fact that
920 the PSF can depend on the orientation of the molecule's dipole, which is generally unknown. If
921 the dipole remains fixed or constrained to certain angles during exposure time, ignoring dipole
922 orientation can bias estimated positions by up to ~125 nm²⁰⁵. Recent work has shown that
923 molecular positions and dipole orientations can be simultaneously estimated in advanced setups
924 that feature polarizers and multiple cameras²⁰⁶. With standard SMLM systems, localization
925 artifacts can fortunately still be avoided using fluorescent labels that rotate rapidly during the
926 exposure time, such that all possible angles are equally sampled and biases are averaged out²⁰⁶.

927 A very different type of artifact are unspecific detections that can arise from autofluorescence or
928 impurities on the coverslip. Autofluorescence is stronger at shorter wavelengths, and can hence
929 be alleviated using longer wavelength (redder) fluorophores and/or appropriate filters. Impurities
930 can be reduced, though not entirely removed, by appropriate cleaning of coverslips, e.g. plasma
931 cleaners²⁰⁷ (**Table 3**).

932 **[H2] Limitations for imaging tissues**

933 Applying SMLM to tissues poses several challenges, some of which can be addressed by adapted
934 sample preparation. A first challenge is fluorescent labeling. Tissues are thicker than cells and
935 must be sufficiently permeabilized to be labelled properly. However, extensive permeabilization
936 can compromise tissue structure. Additionally, fixed thick tissues are more likely to have
937 background fluorescence and increased light scattering. One way to overcome the increased
938 background/scattering problem is to cut tissues into thin slices and reconstruct a 3D SMLM image
939 from many slices²⁰⁸. Cutting thin tissue slices often requires paraffin embedding of the tissue,
940 which can make epitopes unavailable for antibody binding and can decrease labeling efficiency.
941 Antibody labeling prior to embedding and using specific resins for the embedding step such as
942 epoxy-resin can overcome this problem and also maintain high photon output of fluorophores²⁰⁸.
943 Another solution to the fluorescent background problem is tissue-clearing^{209 210}, which together
944 with other optimizations allowed to image high-resolution chromatin using *d*STORM in
945 pathological tissue relevant for cancer diagnosis²¹⁰. In this context, *d*STORM was also shown to
946 be applicable to samples obtained by scalpel excision from tumor tissue²¹¹. Finally, SMLM puts
947 additional demands on the way tissue samples are mounted, since sample drift must be avoided
948 and specialized aqueous imaging buffers are required. To overcome this challenge, an imaging
949 chamber was developed²⁰⁹, consisting of a permeable agarose pad and a custom-built stainless
950 steel imaging adapter to keep tissue slices flat, immobile and bathed in imaging buffer during
951 image acquisition.

952 Optical systems adapted to imaging thick samples and tissue without slicing will be discussed in
953 the 'Outlook' section.

954 [H2] Throughput limitations

955 A major limitation of SMLM techniques is their limited throughput, i.e. the low number of cells
956 imaged per unit time, which results from a relatively small FOV, and the fact that it typically takes
957 minutes or more to obtain a high quality super-resolution image of a single animal cell. This
958 severely restricts the ability of SMLM to image large areas or tissue volumes or to reveal rare
959 cellular phenotypes. Furthermore, SMLM approaches are typically limited to a few colors because
960 of the small number of spectrally distinguishable fluorophores. Nevertheless, several efforts have
961 been deployed to address these limitations:

962 [H3] Larger fields of view

963 Initial SMLM studies were often restricted to a FOV of, say $\sim 25 \times 25 \mu\text{m}$, because they relied on
964 EMCCD cameras with 512×512 pixel arrays, and because non-uniform laser illumination typically
965 results in spatially varying photoswitching and photon emission rates, therefore requiring cropping
966 of images to the central region of this array, where illumination is approximately constant. Much
967 larger FOV can now be obtained using sCMOS cameras with bigger arrays (2024×2024 pixels)
968 and methods to homogenize illumination (flat-fielding)²¹²⁻²¹⁴. For example, a low-cost microlens
969 array (MLA)-based epi-illumination system -flat illumination for field-independent imaging (FIFI)
970 increased the FOV to $100 \times 100 \mu\text{m}^2$ ²¹³, hence allowing to image ~ 2 -5 mammalian cells
971 simultaneously with high resolution.

972 [H3] Multiplexing

973 Several multiplexing approaches have been developed to image more than a few molecular
974 species in the same sample^{215,216}. DNA-PAINT is particularly amenable to multiplexing because
975 DNA sequences provide exquisite programmability and specificity. In Exchange-PAINT²¹⁶,
976 different molecular targets are simultaneously labeled with orthogonal DNA docking strands,
977 followed by sequential delivery, imaging, and washing steps using complementary imager strands
978 (**Fig. 6e,f**). Importantly, each imager species can carry the same dye, since target identity is
979 encoded by the respective sequence and imaging round as opposed to the dye color²¹⁷. Similar
980 approaches can also be used with sequential labeling of proteins by different primary antibodies,
981 using the same dye-conjugated secondary antibodies. For example, maS³TORM (multiplexed
982 automated serial staining stochastic optical reconstruction microscopy) enabled SMLM of 15
983 target proteins in single cells²¹⁵.

984 To increase throughput in multiplexed DNA-PAINT, target molecules can furthermore be
985 engineered to blink with precisely adjustable frequency and duration, thereby providing a distinct
986 "kinetic barcode" for the simultaneous detection of hundreds of unique molecular species²¹⁸. In a
987 related approach, frequency multiplexing DNA-PAINT modulates the excitation lasers at different
988 frequencies and uses Fourier transformation to unmix the fluorescent response, hence enabling
989 simultaneous imaging of several fluorophores²¹⁹.

990 Note that highly multiplexed approaches such as MERFISH or seqFISH+ are now increasingly
991 used to visualize thousands of distinct RNA species or DNA-loci in single cells. Although they rely
992 on sequential labeling rather than photoswitching, these methods enable super-resolution views
993 into 3D genome structure and cellular transcriptome. For more information, see refs.^{146,220}.

994 **[H3] Automation**

995 To perform high throughput or multiplexed imaging for more than a few conditions or target
996 proteins, one needs a fully automated microscope solution that allows fluid exchange, can perform
997 staining, sample positioning and image acquisition. The automation problem is being addressed
998 through several recent developments. Combining high-content screening concepts with SMLM
999 through an automated microscope and automated data analysis, (HCS)–SMLM enabled 3D
1000 imaging of an entire 96-well plate²²¹. The fluid exchange problem was tackled using a fluidics
1001 system composed of low cost Lego hardware controlled by ImageJ software²²². The above-
1002 mentioned maS³TORM used a fully automated and coordinated 3D SMLM microscope with a
1003 pipetting robot to perform staining experiments in situ²¹⁵. An example biological application of
1004 automated SMLM image acquisition and analysis (though not with automated staining) is the
1005 study of 23 proteins involved in yeast endocytosis⁸⁷.

1006 **[H3] Faster SMLM**

1007 Increasing FOV improves throughput, but not the speed and temporal resolution of SMLM, which
1008 typically remain poor. Nevertheless, tremendous progress in optimizing the speed of SMLM has
1009 been made since the first PALM study, which required ~2-12 h for a single FOV. Using sCMOS
1010 cameras with high (>1 kHz) frame rates and synthetic dyes, the rate of localization can be
1011 dramatically increased, although this requires high laser powers (>50 kW cm⁻²) to maintain good
1012 SNR and localization precision^{25,223}. Such optimizations allowed for example 2-color 3D SMLM of
1013 more than 10,000 mammalian cells in ~26 hours²²⁴.

1014 DNA-PAINT has traditionally been a particularly slow SMLM technique. The influx rate of dye-
1015 labeled imagers to their targets, which ultimately determines the achievable acquisition speed, is
1016 limited by the overall DNA association rate k_{on} ($\sim 10^6$ (Ms)⁻¹), leading to a blinking event every
1017 100 s for typical imager concentrations of 10 nM) and the imager concentration. While increasing
1018 the imager concentration can in principle speed-up DNA-PAINT, concentrations in excess of a
1019 few tens of nM would increase background fluorescence, hence reduce SNR and reduce
1020 precision. To address this, FRET-based approaches have been implemented²²⁵⁻²²⁷, effectively
1021 suppressing fluorescence from diffusing imager strands. Orthogonal approaches aim at
1022 increasing k_{on} . This can be done by rational sequence design and buffer optimizations, leading
1023 to an order of magnitude faster k_{on} and thus imaging speed²²⁸. Another approach to increase k_{on}
1024 is based on preloading DNA-PAINT imager strands with Argonaute proteins²²⁹. The latest
1025 development is the use of periodic, concatenated sequence motifs of rationally designed
1026 sequences, which now speeds up DNA-PAINT by 100-fold over the classical implementation²³⁰.

1027 **[H3] Cheaper SMLM**

1028 Although the cost of SMLM hardware, especially custom-built setups, is moderate compared to
1029 other advanced microscopy systems, typical systems still have price tags on the order of
1030 ~100,000 € or more. However, efforts to replace expensive components by cheaper alternatives
1031 (e.g. EMCCDs by sCMOS cameras or scientific grade lasers by LEDs) and/or leverage
1032 smartphone technology have resulted in prototypes ~10 k€ or less^{77,231-233}. Although they do not
1033 always achieve the spatial resolutions of more sophisticated microscopes, such approaches may
1034 prove sufficient for many applications and could also enable large-scale parallelization and hence
1035 gains in throughput.

1036 **[H2] Structural dynamics limitations**

1037 Perhaps the most severe limitation of standard SMLM is its restricted applicability to study
1038 structural dynamics in live cells. Live-cell structural studies are rare, because collecting enough
1039 localizations to provide a super-resolution snapshot of a biological structure before it rearranges
1040 is generally challenging it not impossible. While the above speed optimizations with high frame
1041 rates are applicable to fixed cells, the intense laser excitation required for fast turnover is much
1042 more problematic for live cells because of its adverse effects on cell physiology (phototoxicity),
1043 especially when using UV activation³⁴, and rapid photobleaching. Because of these constraints,
1044 structural SMLM studies have for the most part been limited to technical proofs of principle or to
1045 relatively slowly moving structures with few reconstructed snapshots^{34,85,90,223}. As discussed
1046 above, methods designed for higher activation densities can overcome this limitation to some
1047 extent, but at the cost of a significant reduction in spatial resolution.

1048 **[H1] Outlook**

1049 We conclude this Primer with a look at advanced SMLM techniques and particularly promising
1050 ongoing developments that aim to address some of the main remaining challenges in SMLM.
1051 These include: imaging the ultrastructural context, moving towards truly molecular resolution,
1052 imaging thick samples and tissues, imaging live samples without phototoxicity or photobleaching
1053 and more.
1054

1055 **[H3] SMLM with light sheets**

1056 To overcome the challenges of imaging deep inside thick tissue, SMLM was combined
1057 with light sheet fluorescence microscopy (LSFM) for imaging live²³⁴ and fixed tissues⁸⁹. In LSFM,
1058 the sample is illuminated with a thin light sheet perpendicular to the optical axis²³⁵. This optical
1059 sectioning is advantageous for living tissues, as they experience much reduced irradiation and
1060 hence less stress during imaging, but also for fixed tissues, as background fluorescence is
1061 significantly reduced and contrast is improved. In individual molecule localization-selective plane
1062 illumination microscopy (IML-SPIM), a cylindrical lens is used to create activation and readout
1063 light sheets to photoactivate single fluorescent molecules in thick tissue²³⁴. More recently, lattice
1064 light sheet (LLS) microscopy was combined with 3D dSTORM for plasma membrane receptor

1065 imaging on the basal and apical membrane as well as for 3D single-particle tracking²³⁶ and tissue
1066 imaging by PAINT using newly developed membrane probes⁸⁹, achieving multi-color super
1067 resolution imaging of samples up to 20 μm thick and whole-cell 3D SMLM of intracellular
1068 membranes. The contrast achieved with this approach resembles that achieved with heavy metal-
1069 stained EM images, without mechanical sectioning. Light sheet SMLM can further be combined
1070 with Adaptive Optics (AO) to correct for PSF aberrations induced by the scattering tissue to
1071 improve the imaging depth and spatial resolution when imaging thick samples²³⁷.

1072 [H2] Combining electron microscopy and SMLM

1073 Electron microscopy (EM) generally offers even better spatial resolution than SMLM, and also
1074 provides a global view of the cellular ultrastructure that SMLM does not. Conversely, EM lacks
1075 the molecular specificity afforded by fluorescence. Super-resolution visualization of specific
1076 molecules within an ultrastructural context is possible by combining EM with SMLM on the same
1077 sample (Correlative light and electron microscopy; CLEM). For example, combining platinum
1078 replica electron microscopy (PREM) (a technique where the sample is coated by a heavy metal
1079 to increase its contrast by EM), with iPALM and STORM, provided important details about the
1080 distinct structural zones inside clathrin-coated structures^{238,239}. Similarly, SMLM and PREM
1081 allowed to show that actin rings found in neurons consist of long, intertwined actin filaments,
1082 revealing an unexpected braid-like organization of actin rings²⁴⁰. Correlative cryogenic SMLM
1083 (cryo-SMLM) and electron tomography was demonstrated to study the subcellular localization of
1084 important regulatory proteins in *Caulobacter crescentus*²⁴¹. In a recent tour de force, fluorescently
1085 labeled high-pressure frozen cells were preserved in vitreous ice, imaged with cryo-SMLM and
1086 subsequently with 3D focused ion beam scanning electron microscopy (FIB-SEM), and the
1087 images resulting from the two modalities were registered to nanoscale precision. This approach
1088 achieved $\sim 40\text{nm}$ resolution visualization of protein distribution within frozen whole cells in 3D in
1089 the context of their ultrastructure²⁴² and revealed unexpected relationships between different
1090 cellular compartments, such as the existence of ER proteins inside the nucleus. These methods,
1091 while requiring challenging sample preservation and preparation, nonetheless hold great promise
1092 for visualizing various molecular complexes in their ultrastructural context.

1093 [H2] SMLM with opposing objectives

1094 Because localization precision critically depends on the SNR, one way to improve resolution is to
1095 collect more photons from single fluorophores. This can be done using two opposing objectives
1096 (4 Pi setups) that collect twice as many photons from single fluorophores as single objective
1097 systems, thus improving localization precision by a factor $\sim\sqrt{2}$. Such systems enabled lateral
1098 resolutions $<10\text{nm}$ and, in combination with astigmatism (**Box 4**) axial resolution $<20\text{nm}$ in 3D
1099 STORM, allowing to distinguish individual actin filaments and to visualize two distinct layers of
1100 actin networks that showed sheet-like protrusions²⁴³. Interferometric PALM (iPALM)²⁴⁴ also uses
1101 two opposing objectives, but allows laser beams to interfere in a three-way beam splitter and
1102 propagate to 3 CCD cameras, allowing to determine the 3 coordinates of fluorescent molecules.
1103 In contrast to PSF shaping or multi-plane systems (see **Box 4**), iPALM does not trade off
1104 localization precision for axial range and achieved isotropic $<20\text{ nm}$ 3D resolution with fluorescent

1105 proteins. More recently, interferometric SMLM has been extended to achieve 10-20 nm resolution
1106 imaging of the ER, mitochondria, NPCs, bacteriophages and other structures in whole mammalian
1107 cells without compromising resolution²⁴⁵. Careful calibration of refractive index variations will be
1108 needed to extend this approach to whole nuclei.
1109

1110 **[H2] SMLM with minimum fluxes**

1111 Since the onset of SMLM, the dominant paradigm to increase localization precision has been to
1112 collect more photons from single fluorophores, as in the methods above. This paradigm has
1113 meanwhile been shifted by the introduction of MINFLUX²⁴⁶, a concept for localizing single emitters
1114 not by their highest intensity, but by lowest emission fluxes arising from a local minimum in
1115 excitation. In the original implementation, an excitation doughnut in combination with a three-
1116 point-estimator was used to precisely determine the emitter position (**Fig. 7a**). In combination with
1117 photoswitching, MINFLUX attains single nanometer resolution with considerably reduced photon
1118 counts compared to standard SMLM (**Fig. 7b**). This allows for very high localization precisions,
1119 even with relatively dim FPs, and >100-fold faster single molecule tracking than previous
1120 methods²⁴⁶.

1121 Recently, two independent groups have replaced the excitation doughnut by a standing
1122 wave to spatially modulate excitation intensities, either using diffraction gratings (SIMFLUX²⁴⁷) or
1123 optical interference (Repetitive Optical Selective Exposure or ROSE²⁴⁸). In both implementations,
1124 localization precision is improved by ~2-fold compared to standard SMLM. While MINFLUX can
1125 theoretically achieve arbitrarily high localization precision, SIMFLUX and ROSE possess the
1126 advantage that image acquisition times do not scale with FOV, thus currently offer faster
1127 acquisition speed.

1128 With further advancements (e.g. 3D, multicolor, live cell imaging)²⁴⁹ and simplification to
1129 hardware and software, these methods are poised to set a new standard for molecular-resolution
1130 SMLM.

1131 **[H2] SMLM in expanded samples**

1132 Whereas almost all super-resolution methods aim to diminish the size of the effective
1133 PSF², expansion microscopy (ExM) takes an entirely orthogonal approach and improves
1134 resolution by physically expanding a sample embedded in a polyelectrolyte hydrogel²⁵⁰ (**Fig. 7c**).
1135 Although ExM can achieve super-resolution with conventional microscopes (e.g. 70 nm resolution
1136 with five-fold expansion²⁵⁰ or 25 nm with 20-fold expansion²⁵¹), combining it with SMLM can
1137 potentially improve resolution down to single nanometers. An important challenge is that many of
1138 the targeted molecules can lose their label after free-radical polymerization of the hydrogel,
1139 compromising labeling density and resolution²⁵². Furthermore, addition of photoswitching buffer
1140 as required for dSTORM leads to shrinking of polyelectrolyte hydrogels. Finally, expansion of
1141 prelabelled samples results in further displacement of the fluorophore from the target molecule,
1142 increasing the linkage error, e.g. from 17.5 nm with indirect immunostaining to 70 nm after 4-fold
1143 expansion, again severely limiting the resolution. Recently, trifunctional linkers have been
1144 developed that are inert to polymerization, digestion and denaturation, and enable direct labeling
1145 and covalent linking of target molecules and functional groups to the hydrogel^{253,254}. Furthermore,

1146 it was shown that re-embedding of the expanded hydrogel prevents shrinking and post-expansion
1147 labeling preserves the ultrastructure of multiprotein complexes, improves the labeling efficiency
1148 and reduces the linkage error from 17.5 nm to ~5 nm for 3.2-fold expansion²⁵⁵. The potential of
1149 these new methods has been demonstrated by imaging of clathrin-coated pits, microtubules and
1150 centrioles with ~5 nm resolution^{255 254} (**Fig. 7d**). Ex-SMLM thus provides another promising road
1151 map for achieving true molecular resolution.

1152 **[H2] Enhanced SMLM with deep learning**

1153 The increasingly complex imaging data acquired thanks to advances in SMLM exacerbate the
1154 demand for computational analysis. In recent years, the field of computer vision has been
1155 revolutionized by machine learning methods based on multi-layer artificial neural networks (deep
1156 learning)²⁵⁶. Unsurprisingly, deep learning is now also adapted to advance light microscopy
1157 methods, including SMLM²⁵⁷. For example, deep learning has been used to extract single
1158 molecule localizations from diffraction-limited images, including in high activation density
1159 conditions, which can dramatically reduce processing time compared to MLE²⁵⁸. However, deep
1160 learning can also help accelerate image acquisition itself by overcoming apparently fundamental
1161 sampling constraints. This is possible because deep learning can reconstruct high quality images
1162 from much fewer localizations than traditionally needed²⁵⁹ by learning structural redundancies
1163 from similar images (**Fig. 7e**). With further adaptations, such methods might greatly facilitate
1164 super-resolution in live cells while minimizing phototoxicity, as recently demonstrated for 3D light
1165 sheet microscopy²⁶⁰. Deep learning will certainly also make inroads in the quantitative analysis of
1166 SMLM data, such as the segmentation of biological features^{261,262}.

1167 The power of deep neural networks comes from their ability to learn complex features from
1168 training data. However this reliance on training data also carries the risk that neural network
1169 outputs might be biased towards the training data, potentially leading to miss novel phenotypes
1170 or even to generate artifacts^{257,259,263}. This well-known Achilles heel of machine learning therefore
1171 calls for careful validations and/or retraining of existing deep learning frameworks and the
1172 development of methods that are aware of or robust to mismatches between testing and training
1173 data. If these challenges are correctly addressed, machine learning methods will likely play a key
1174 role in overcoming key limitations of advanced microscopy and SMLM in particular.

1175

1176 **[H2] Concluding remarks**

1177 SMLM is a powerful, yet relatively accessible, family of imaging technique that combines
1178 fluorophore chemistry, optics and computation to image and analyze biological structures and/or
1179 dynamics at increasingly higher resolution. In this Primer, we attempted to introduce all main basic
1180 aspects of SMLM, highlighting biological applications that illustrate the maturity of SMLM
1181 methods, while also covering advanced and promising recent developments. As the SMLM field
1182 continues to evolve, it brings us nearer to a future where all molecules within a cell, tissue or
1183 organism may be individually localized, counted and tracked. We hope that this Primer will
1184 encourage more investigators to adopt and further improve SMLM to explore the innermost
1185 architecture and mechanisms of living systems.

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1189 **TABLES**

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1191 **Table 1: Selected commercial reagents for SMLM experiments**

1192 This table lists examples of some recommended reagents for newcomers to SMLM. For multi-color imaging,
 1193 see **Box 2**. For more comprehensive information and/or systematic comparisons of SMLM labeling
 1194 techniques, see e.g. ^{35,36,60,264,265} and the protocols listed in **Table 3**.

Type of reagent	Reagent(s)	Supplier	Reference	Application
Primary antibodies, unlabeled (use with labeled secondary antibodies)	Rat anti-tubulin antibody (recognizes the α subunit of tubulin)	Bio-Rad	link	(d)STORM of microtubules
	Rabbit anti-nucleoporin Nup133	Abcam	link	(d)STORM of nuclear pores in human cells
Secondary antibodies conjugated to synthetic dyes (use with unlabeled primary antibodies)	Anti-rat antibody coupled to Alexa Fluor 647	ThermoFisher	link	dSTORM
	Anti-rabbit antibody coupled to Alexa Fluor 647	Jackson ImmunoResearch	link	dSTORM
Fluorescent protein plasmids	mEos3.2 plasmid	Addgene	link	(F)PALM
	mMaple3 plasmid	Addgene	link	(F)PALM
DNA-PAINT kits	DNA-conjugated secondary antibodies/nanobodies (docking strands)	Massive Photonics	link	DNA-PAINT
	Dye-conjugated DNA (imager strands)	Massive Photonics	link	DNA-PAINT
Direct labeling	Phalloidin conjugated to Alex Fluor 647	ThermoFisher	link	dSTORM of actin
	MitoTracker Red	ThermoFisher	link	Live cell STORM of mitochondrial membranes
Photoswitching buffers	SAFe Reagents	Abbelight	link	(d)STORM
	Everspark	idylle	link	(d)STORM
Labeled samples	Cells with labeled microtubules, mitochondria, podosomes..	Abbelight	link	(d)STORM
Fiducial markers	TetraSpeck microspheres (100 nm diameter)	ThermoFisher	link	Correction of drift and/or chromatic aberrations

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1196 **Table 2: Selected SMLM software**

1197 This table lists a few recommended software packages for SMLM image reconstruction. All listed software
1198 are free, can reconstruct 3D SMLM images and all except SMAP enable drift correction. This selection is
1199 partly subjective, as it is based on software known to (and in part developed by the authors), but also based
1200 on objective results from the 2D/3D SMLM localization challenges. For more details and quantitative
1201 comparisons of these and many other SMLM software (currently 95), we refer to the challenges and their
1202 updates (<http://bigwww.epfl.ch/smlm>).

Software name	URL	Comments	Reference
ThunderSTORM	https://zitmen.github.io/thunderstorm/	Widely used, implements multiple 2D and 3D algorithms. Strong performance in 2D SMLM challenge	94
SMAP	https://github.com/jries/SMAP	Handles arbitrary PSFs; Strong performance in 3D SMLM challenge	266,267
SMOImphot	https://bitbucket.org/ardiloot/smolphot-software/wiki/Home	Strong performance in 3D SMLM challenge	NA
ZOLA-3D	https://github.com/imodpasteur/ZOLA-3D	Handles arbitrary PSFs and refractive index mismatch	102
Picasso	https://github.com/jungmannlab/picasso	Software package optimized for DNA-PAINT	19

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1206 **Table 3: Selected experimental protocols for SMLM**

1207 The following is a non exhaustive list of some recommended experimental protocols for SMLM.

- 1208 • Van de Linde et al. (ref. ²⁷) : *d*STORM, live cells.
- 1209 • Gould et al. (ref. ²⁰²) : (F)PALM.
- 1210 • Schnitzbauer et al. (ref. ¹⁹): DNA-PAINT, multicolor, quantification (qPAINT).
- 1211 • Jimenez et al. (ref. ²⁶⁸): *d*STORM, DNA-PAINT, multicolor, optimized sample preparation.
- 1212 • Manley et al. (ref. ²⁶⁹): spt-PALM.
- 1213 • Kaplan et al. (ref. ²⁷⁰): sample preparation optimized for *d*STORM in yeast.
- 1214 • Davis et al. (ref. ²⁰⁷): how to prepare coverslips to minimize impurities in SMLM

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1219 **FIGURE LEGENDS**

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1221 **Figure 1: Principle of SMLM.**

1222 **a)** A single fluorescent molecule (green dot) imaged through a microscope appears on the camera
1223 as a fuzzy, ~200 nm wide spot (the PSF) extending over multiple pixels. **b)** PSFs from
1224 simultaneously emitting molecules overlap if their distances are smaller than the PSF. **c)** The
1225 (X_0, Y_0) coordinates of a single molecule can be computed with high precision, because subpixel
1226 displacements (e.g. here by 0.5 pixels in X and Y) lead to predictable changes in pixel values, as
1227 shown by the grey scale image (bottom) and corresponding 2D histogram (center) (simulated
1228 data). The mesh surface shows a Gaussian model PSF centered on (X_0, Y_0) . **d)** Higher photon
1229 counts (N) lead to higher SNR images and more precise localizations (red crosses). **e)** SMLM
1230 usually exploits the fact that fluorophores stochastically switch between an active ('ON') state and
1231 one or more inactive ('OFF') states. **f)** A diffraction-limited image of nuclear pores, with all
1232 fluorophores ON, is blurred by overlapping PSFs. **g)** A sequence of diffraction-limited images of
1233 the same area, where only few molecules are ON simultaneously. **h,i)** In each frame, single
1234 molecules are computationally detected (**h**) and localized (**i**). Molecules at the border of the image
1235 are not detected. **j)** An SMLM experiment results in a localization table, where each row
1236 represents a distinct localization event and columns indicate x, y coordinates and additional
1237 information, e.g. frame number and photon counts (N). There are usually multiple localizations
1238 per frame, and the same molecule can be localized in multiple frames. **k,l)** Accumulated
1239 localizations visualized as a scatter plot (**k**), or a 2D histogram (**l**), with bins much smaller than
1240 raw image pixels. The raw image pixels are shown by the dashed grid, and 10x10 small bins are
1241 shown inside a single raw image pixel in **k**). This 'super-resolution' image reveals the ring-like
1242 (octagonal) structure of nuclear pores that was previously hidden.

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1244 **Figure 2: Fluorophore types and labeling strategies in SMLM.**

1245 **a-e)** Fluorophores compatible with SMLM can be divided into five classes: photoswitchable (**a**),
1246 photoactivatable (**b**), photoconvertible (**c**), spontaneously blinking (**d**), or temporarily binding (**e**).
1247 The boxes indicate properties of fluorophores in each class and an example fluorophore. The
1248 schematic involving the lambda symbol indicates the nature of switching that enables SMLM. **f)**
1249 Comparison of different fluorescent labeling approaches and the resulting linkage error, i.e. the
1250 distance between the molecular target of interest (here, tubulin) and the fluorophore. From left to
1251 right: immunolabeling with primary and secondary antibodies (orange); labeling with a small
1252 camelid antibody (nanobody, yellow), often in combination with primary antibodies or GFP
1253 (green); labeling with a genetically encoded protein such as Eos (magenta) or a self-labeling
1254 protein tag; direct labeling with a dye-conjugated ligand such as the microtubule-binding
1255 compound docetaxel (turquoise box); incorporation of unnatural amino acids such as TCO*-lysine
1256 (blue box) via genetic code expansion (GCE) enables rapid labeling with functionalized synthetic
1257 dyes. Structure of immunoglobulin (PDB-ID 1IGT), green fluorescent protein bound to enhancer
1258 nanobody (PDB-ID 3K1K), EOS-FP (mEos2, PDB-ID 3S05), exemplified target protein (tubulin,
1259 PDB-ID 1FFX).

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1261 **Figure 3: SMLM Hardware.**

1262 **a,b)** A basic SMLM setup consists of illumination (laser, Köhler lens, i); imaging optics and stage
1263 (objective, sample, ii) and detection (tube lens, camera, iii). Dichroic mirrors are used to separate
1264 excitation and emission wavelengths, and can be combined with additional emission filters to
1265 reject autofluorescence. **c)** A 2D *d*STORM image of microtubules. **d)** A 3D SMLM system can be
1266 obtained simply by adding an optical component to engineer the PSF, such as a cylindrical lens,
1267 which generates astigmatism. **e)** A z-stack of a fluorescent bead shows the axial variations of an
1268 astigmatic PSF. **f)** A 3D super-resolution image obtained by analysis of 2D single molecule
1269 images, displayed here in 2D with color indicating z. Here, PSF calibration and image
1270 reconstruction was performed with ZOLA-3D¹⁰².

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1272 **Figure 4: Live cell SMLM**

1273 **a)** Structural dynamics of a focal adhesion (tdEos-paxillin) reveal its appearance near the cell
1274 edge before maturation and motion toward the interior. Each super-resolution image is
1275 reconstructed from 1,000 raw frames. Scale bar: 500 nm. From ref. ³⁴. **b)** Molecular dynamics of
1276 the vesicular stomatitis virus glycoprotein (VSVG-tdEos), a transmembrane protein freely
1277 diffusing on the plasma membrane. The motion of each protein is traced over multiple frames,
1278 with different colors representing individual molecules (left). Each trajectory can be analysed, to
1279 create a map of diffusion coefficients (center). In contrast, the dynamics of molecules within the
1280 actin cytoskeleton (actin-tdEos) show directed motion near the cell's leading edge and diffusive
1281 motion toward the interior (right). Scale bars: 500 nm. From ref. ¹⁶.

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1283 **Figure 5. Major discoveries enabled by SMLM**

1284 **a)** STORM image of histone H2B in human fibroblast cells (hFb) with progressively higher zoomed
1285 insets, adapted from ref.¹³⁹. Scale bars: 2 μ m and 500 nm. **b)** Top: *d*STORM image of nuclear
1286 pore complexes (NPCs) labeled with antibodies against the nucleoporin Nup133. Three individual
1287 NPCs are shown on the right and an average image of 4,171 aligned NPCs on the lower right.
1288 Scale bars: 0.5 μ m and 0.1 μ m. Bottom: Left: Colored circles show radial positions of different
1289 nucleoporins in the plane of the nuclear envelope, determined from averaged images, with the
1290 inferred position of the Y-shaped scaffold complex overlaid. Circle thickness reflects 95%
1291 confidence intervals of average radial distances. Center and right: side and frontal views of the
1292 EM structure (grey), with the radial positions of nucleoporins shown in color, and two positions of
1293 the Y complex consistent with the data overlaid. Scale bar, 40 nm. From ref.¹²⁰. **c)** iPALM image
1294 of a human U2OS cell expressing integrin α_v -tdEos (left) and actin-mEos2 (right) with color coded
1295 zoomed insets of boxed regions adapted from¹⁶⁹. Colors represent the z-position relative to the
1296 substrate ($z=0$ nm). Scale bars: 500 nm. **d)** 3D STORM image of actin in a neuronal axon with
1297 zoomed y/z insets of boxed regions showing actin rings, adapted from⁵⁸. Scale bars: 1 μ m and
1298 200 nm.

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1300 **Figure 6: Limitations and optimizations**

1301 **a)** SMLM image of microtubules before and after drift correction. Arrows show a fluorescent bead
1302 used to estimate the drift. **b,c)** Artifacts due to PSF overlaps in simulated images. **b)** Left: ground
1303 truth image without localization errors, shown as a scatter plot. Middle: SMLM image for a low
1304 density of activated fluorophores (10 localizations per μ m², no PSF overlaps). Right: the same,
1305 for a high activation density (50 localizations per μ m²); overlapping PSFs cause artifactual

1306 localizations near the center. **c)** Simulated molecular clusters, with a 10-fold higher density for the
1307 top cluster. Left: simulated ground truth shown as scatter plot. Middle: SMLM image without
1308 filtering. Right: the same after filtering of poor localizations due to overlapping PSFs. After filtering,
1309 the high density cluster is barely visible. **d)** Artifacts in SMLM images of microtubules resulting
1310 from subpixel localization bias. Top: without bias. Bottom: with bias; the bias is due to
1311 computational localization with an incorrect PSF model. As a result of bias, the reconstructed
1312 image shows a grid pattern. Insets show the entire FoV. The localization bias is readily apparent
1313 in the histogram of x coordinates relative to the center of pixels in the raw images (drift correction
1314 was not applied to better highlight the effect of localization bias). **e)** Exchange-PAINT implements
1315 sequential imaging of multiple targets by DNA-PAINT with different imager strands labeled with
1316 the same dye. The sample is first labeled with orthogonal docking strands P_1, P_2, \dots, P_n , then the
1317 first imager strand species P^*_1 , complementary to docking strand P_1 , is introduced and a DNA-
1318 PAINT image of P_1 is acquired. Next, the strands P^*_1 are washed out, then imager strands P^*_2
1319 are introduced and a DNA-PAINT image of P_2 is acquired, etc. for n cycles. Each DNA-PAINT
1320 image is assigned a distinct pseudocolor and all n images are then superposed. **f)** Pseudocolor
1321 DNA-PAINT images of origami structures displaying digits 0–9. Scale bar: 25 nm.

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1324 **Figure 7: New directions in SMLM**

1325 **a,b)** MINFLUX. **a)** MINFLUX excitation concept to precisely probe emitter positions by minimal
1326 photon fluxes. A doughnut-shaped excitation (green) is moved sequentially to four probing
1327 positions r_0, r_1, r_2, r_3 (colored circles) in the vicinity of a single fluorophore (orange star). The
1328 probing range is given by L . If the doughnut center coincided perfectly with the fluorophore
1329 position, no photons would be emitted. The position of the fluorophore can be calculated with very
1330 high precision from the fluorescence photon counts shown below. From ref.²⁴⁶. **b)** Example
1331 nuclear pore complex imaged by MINFLUX. Scale bars: 50 nm. From ref.²⁴⁹ **c,d)** Expansion-
1332 SMLM. **c)** In expansion microscopy (ExM), samples are embedded in a gel that expands upon
1333 hydration. Immunolabeling of epitopes can be performed before or after gelation and expansion
1334 using linkers that bind to the gel and to a fluorophore. Full or partial protein digestion is commonly
1335 used to enable isotropic expansion. In order to enable (d)STORM imaging in photoswitching
1336 buffer the sample is re-embedded in an uncharged polyacrylamide gel after expansion. **d)** Left:
1337 3D post-labeling Ex-dSTORM image of a 3.2x expanded and re-embedded sample shows the
1338 ninefold symmetry of the procentriole. Scale bar, 500 nm. Right: 3.1-fold expanded and re-
1339 embedded tubulin filaments. Magnified view of highlighted region (white box). xz side view cross
1340 section of a tubulin filament showing its hollow structure. Scale bars, 2 μm (large square), 500 nm
1341 (vertical rectangle), 200 nm (small square). From ref.²⁵⁵ **e)** Deep learning accelerates SMLM
1342 image acquisition. A widefield image (WF) and a sparse SMLM image obtained from $k=300$
1343 frames only are fed as inputs to an artificial neural network (ANN) that was previously trained on
1344 high quality (long acquisition) SMLM images of microtubules. The ANN outputs a super-resolution
1345 image that is in good agreement with an SMLM image obtained from $k=30,000$ frames ('ground
1346 truth'), suggesting a 100-fold reduction in acquisition time without compromising spatial resolution.
1347 Adapted from ref.²⁵⁹.

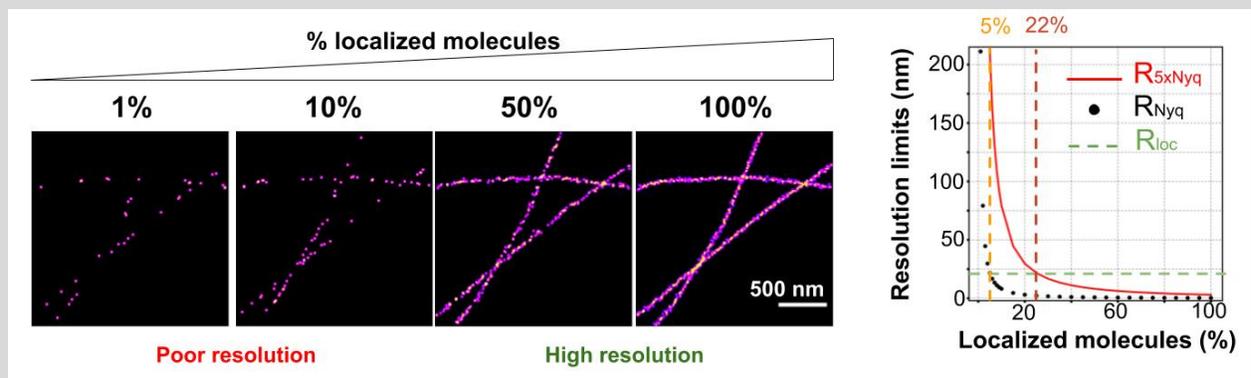
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1349 **BOXES**
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1351 **Box 1: The resolution of SMLM images**

1352 Determining the resolution of an SMLM image is not straightforward. The resolution R cannot be
1353 better than $R_{loc} \approx 2.3 \sigma_{loc}$, thus estimating localization precision σ_{loc} is key. One approach to
1354 estimate σ_{loc} is to calculate the Cramer-Rao lower bound (CRLB), but this estimate is generally
1355 too optimistic. More realistic estimates can be obtained from the standard deviations of
1356 coordinates in small localization clusters likely originating from single molecules. However, both
1357 estimates ignore potential localization biases or linkage errors. A common alternative is to
1358 calculate the dispersion of coordinates (e.g. the full width half maximum, or FWHM) across thin
1359 structures such as cytoskeletal filaments. This provides a conservative upper limit to $2.3 \sigma_{loc}$, since
1360 the FWHM reflects both precision and the width of the labeled structure (including linkage errors).

1361 A critical, but often overlooked requirement to achieving a resolution $R_{loc} = 2.3 \sigma_{loc}$ is to have a
1362 good sampling, i.e. a sufficiently high percentage of localized molecules. An early study
1363 introduced a Nyquist sampling criterion to calculate the sampling limit (R_{Nyq}) to resolution based
1364 on the nearest neighbor distances between localizations³⁴, but a reanalysis suggested that 5-fold
1365 higher sampling (R_{5xNyq}) is actually required⁸⁹. In the simulated images below, with $\sigma_{loc} = 10$ nm,
1366 this criterion implies that 22% of molecules must be localized to achieve a resolution $R_{loc} = 23$
1367 nm. A Fourier Ring Correlation (FRC) criterion²⁷¹ — which measures the correlation between
1368 subsets of localizations — simultaneously accounts for localization precision and sampling, but
1369 requires a user-defined threshold and can hide spatial variations in resolution. More recently, an
1370 alternative, threshold-free approach based on phase correlation was proposed²⁷².



1371 While useful, such measures are insufficient to rigorously determine the resolution. Compelling
1372 evidence that an image has resolution R or better is to clearly distinguish distinct structures at
1373 distances $\leq R$. In biological samples, this is done most convincingly on complexes where
1374 molecules are located at known distances from each other, such as nuclear pores, which have
1375 therefore become a gold standard to evaluate resolution¹¹³.
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BOX 2: Multicolor SMLM

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The investigation of biomolecular interactions in cells requires super-resolution imaging of multiple target molecules in one experiment. Multicolor SMLM with synthetic dyes can be accomplished using the classical STORM concept with probes that contain both an activator and a reporter fluorophore⁸. Here, various activator-reporter dye pairs attached to an antibody, such as Alexa Fluor 405 & Cy5, Cy2 & Cy5, and Cy3 & Cy5, enable multicolor STORM using three different activation lasers and spectrally-selective activation of the reporter fluorophore Cy5²⁷³.

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Activator-free SMLM with synthetic dyes (*d*STORM) requires fluorophores that exhibit similar blinking efficiency under identical photoswitching buffer conditions. Suitable pairs for two-color *d*STORM include Alexa Fluor 532 & Alexa Fluor 647, ATTO 520 & Alexa Fluor 647, and CF 568 & Alexa Fluor 647, as well as combinations with longer-wavelength absorbing dyes, e.g. DyLight 750^{152,274}.

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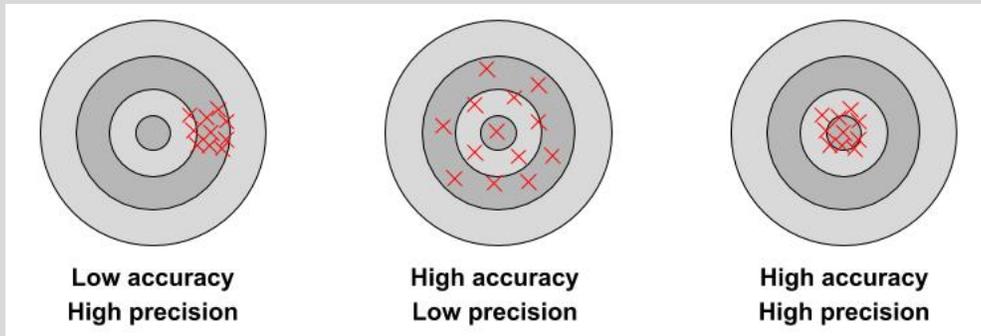
To avoid chromatic aberrations, multicolor SMLM by spectral-demixing can be used. Here, one can use synthetic dyes that exhibit good photoswitching performance in the same thiol switching buffer and can be efficiently excited with the same laser wavelength but exhibit different emission maxima. The emission light is spectrally separated by a dichroic beamsplitter and imaged onto two cameras or separate parts of the same camera chip. The fluorophores are then classified by their emission ratio. Alternatively, the emission signal of different synthetic dyes can be split into two detection paths and recorded simultaneously on two cameras (or two regions of the same camera): one emission spectra recording and one for localization²⁷⁵⁻²⁷⁷. FPs have also been used successfully for multicolor SMLM, either alone, e.g. Dronpa & mEos, or in combination with synthetic fluorophores, e.g. mEos2 & Alexa Fluor 647 or Dronpa & Alexa Fluor 647^{177,278}.

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Box 3: Localization precision, accuracy and the Cramer-Rao lower bound

The image I of a single fluorophore is the result of a stochastic process that depends on several unknown parameters, notably the (x, y) coordinates of the molecule and the number of collected photons, N . Localization algorithms attempt to determine (x, y) from I and provide estimates (\hat{x}, \hat{y}) . Algorithms are usually assessed based on their mean squared error (MSE): $MSE(\hat{x}) = \langle (\hat{x} - x)^2 + (\hat{y} - y)^2 \rangle$ where brackets denote statistical averaging. The MSE for coordinate x can be rewritten: $MSE(\hat{x}) = Var(\hat{x}) + B^2(\hat{x})$, where the variance $Var(\hat{x}) = \langle (\hat{x} - \langle \hat{x} \rangle)^2 \rangle$ is the random component of the errors, while the bias $B(\hat{x}) = x - \langle \hat{x} \rangle$ is the systematic error (and likewise for y). The variance defines the ‘precision’ of an algorithm, while the bias defines the ‘accuracy’. High precision algorithms tend to provide similar coordinates for different images of the same molecule, but these may have a large offset (bias) from the true value. High accuracy algorithms tend to find the correct position on average, but possibly with high dispersion (low precision), implying that individual localizations may still be far from the true location.



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One usually considers algorithms without bias, so that $MSE(\hat{x}) = Var(\hat{x})$. The Cramer-Rao lower bound (CRLB) provides a lower limit to the variance (and hence precision) of any unbiased algorithm. Assuming for simplicity that the image depends only on x , the CRLB reads: $Var(\hat{x}) \geq - \left[\left\langle \frac{\partial^2}{\partial x^2} \ln p(I; x) \right\rangle \right]^{-1}$ where $p(I; x)$ is the probability of observing I if the true coordinate is x ($p(I; x)$ is also called the likelihood). A handy formula can be derived under strongly idealized conditions, where the point spread function (PSF) is modeled as a Gaussian of standard deviation σ_0 , the background and camera noise are neglected, and the exact coordinates of the N photons hitting the camera is recorded (i.e. ignoring pixelation). Under these assumptions, the CRLB reduces to: $Var(\hat{x}) \geq \sigma_0^2/N$, and the precision limit becomes: $\sigma_{loc} = \sigma(\hat{x}) \geq \sigma_0/\sqrt{N}$.

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BOX 4: Three-dimensional (3D) SMLM

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Biological structures are generally three-dimensional (3D), and should therefore be imaged in 3D with axial resolutions similar to those that can be achieved laterally. Innovations in optical setups exploiting point spread function (PSF) engineering^{80,279} or multi-plane^{280,281} detection have enabled precise localization of molecules in 3D, in samples up to several micrometers thick¹⁰².

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Methods with engineered PSFs exploit the fact that the 2D image of a fluorescent molecule depends not only on its lateral (x, y) coordinates but also on its axial coordinate z . By analyzing the 2D image pattern, the z coordinate can be estimated in addition to x and y . In this way, 3D super-resolution images can be reconstructed from 2D images obtained without refocusing. The most common approach exploits an astigmatic PSF, generated by inserting a single cylindrical lens into the imaging path^{79,80,102} (see Figure). Other PSFs such as double helix (Figure), tetrapod or saddle point, which can be obtained using phase masks, deformable mirrors or other optical devices, can achieve larger axial range^{102,279,282,283}.

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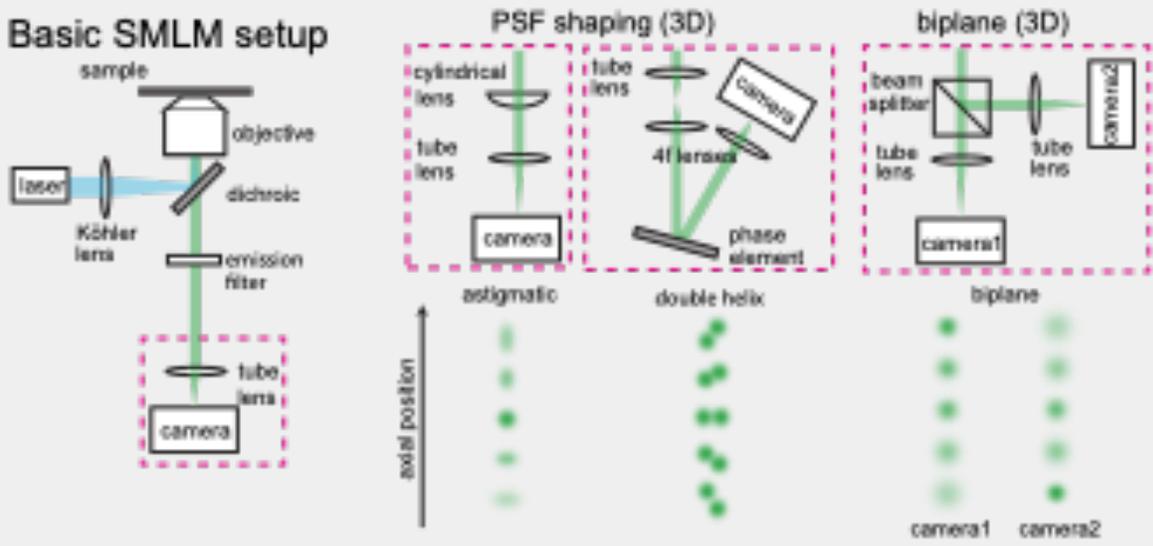
In setups with two or more focal planes (see Figure)^{280,281,284}, analyzing the relative intensities in different images of the same molecule also allows to compute z in addition to (x, y) . In multifocal microscopy (MFM), a specialized diffractive grating is used to image several (e.g. up to 9) focal planes simultaneously on a single camera²⁸¹. Combining MFM with SMLM allowed fast volumetric imaging of mitochondria in whole cells with high resolution²⁸².

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PSF shaping or multi-plane detection divert photons for 3D encoding, resulting in a loss of localization precision. The theoretically achievable localization precision and axial range depend on the PSF shape, and can be calculated. Some PSFs have even been engineered to achieve theoretically optimal precisions over a given axial range^{99,283}. Many software packages are now available for 3D SMLM with PSF engineering⁹⁹.

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1464 **Competing interests**

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1466 Ralf Jungmann is cofounder of Ultivue, Inc. and Massive Photonics GmbH. Christophe Zimmer
1467 has an ongoing collaboration with Abbelight. All other authors declare no competing interests.

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1469 **Glossary**

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- 1471 • **Diffraction:** bending of light waves at the edges of an obstacle such as an
1472 aperture.
- 1473
- 1474 • **Point spread function (PSF):** the image of an infinitesimally small light source
1475 through the optical system.
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- 1477 • **Airy pattern:** a pattern of light featuring a central bright disk surrounded by
1478 increasingly dimmer concentric rings formed by diffraction from a circular
1479 aperture.
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- 1481 • **Total internal reflection (TIRF):** a configuration in which a strongly inclined
1482 laser beam is reflected by the coverslip, leaving only a thin (~200 nm) layer from
1483 the coverslip illuminated, strongly reducing background.
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- 1485 • **Highly inclined and laminated optical sheet (HILO):** a configuration in which
1486 the laser beam enters the sample at a sharp angle, allowing to reduce the
1487 background when imaging at a distance from the coverslip.
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- 1489 • **Noise:** Random fluctuations of the image pixel values.
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- **Poisson noise (shot noise):** A type of statistical noise affecting photon counts and arising from the fact that photons are hitting pixels independently of each other with constant probability per unit time.
 - **Cramer-Rao lower bound (CRLB):** A fundamental limit to the precision (variance) of any estimator without bias.
 - **Ripley's K function:** A function computed from a set of points that helps reveal if points are uniformly randomly distributed or exhibit spatial clustering or dispersion.
 - **Nyquist sampling:** sampling a continuous signal at twice the highest frequency allows to reconstruct it without loss of information.
 - **Fourier ring correlation (FRC):** a measure of image resolution computed from the cross-correlation between two independent noisy versions of the image in Fourier (frequency) space.

1511 **Related links:**

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- Single-Molecule Localization Microscopy Software Benchmarking: <http://bigwww.epfl.ch/smlm>
 - Zenodo: an open access repository for research software and data: <https://zenodo.org/>
 - FigShare: <https://figshare.com/> an open access repository for figures, data sets and images
 - Image Data Resource <https://idr.openmicroscopy.org/> a public repository of imaging data

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