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Single Molecule Microscopy meets Deep Learning

The optical microscope is a natural choice for observing life on the micro-scale, enabling non-destructive investigation of cells, tissue and microorganisms, often in conditions that resemble their natural environments. Indeed, optical microscopy is widespread in biological research. Specifically, fluorescence imaging is common in microscopy, because it enables imaging with high signal-to-background ratio and specificity, which translates to seeing only the structures/proteins of interest. However, optical microscopy comes with a fundamental limitation: due to the wave nature of light propagation, the best achievable resolution of an image-forming optical microscope is somewhat better than half the wavelength of the light; features smaller than ~200-300 nm cannot be resolved.

Technological developments in recent decades, relying on an element of optical and/or chemical innovation, always combined with an appropriate computational algorithm, have been tremendously successful in surpassing this resolution limit. Notable techniques include Stimulated Emission Depletion (STED [1]), Structured Illumination Microscopy (SIM [2]), Single Molecule Localization Microscopy (SMLM [3], [4]), and related variants [5]. The development of super-resolved fluorescence microscopy was acknowledged by the 2014 Nobel Prize in Chemistry awarded to Eric Betzig, Stefan Hell and W.E. Moerner, and using such approaches, resolution improvement by a factor of 10 and more is nowadays attainable in microscopes at research labs and by commercially available instruments.

Specifically, SMLM is a super-resolution microscopy method that is appealingly simple, in terms of instrumentation requirements, requiring only a standard high-NA microscope and a computer. It works by capturing a sequence of standard (diffraction-limited) images of a fluorescently labeled sample, where at each point in time, only a small subset of the typically millions of molecules labeling the sample are emitting light, via one of various mechanisms [3], [4], [5], [6]. The end result is a movie, typically consisting of thousands of frames, containing random spots (Fig. 1a). Then, to obtain super-resolution, each spot is localized, i.e., its x-y position is found algorithmically, which can be done at very high precision (tens of nm), assuming each spot originates from a single molecule. This list of localizations can then be rendered into a single super-resolution image (Fig. 1b).

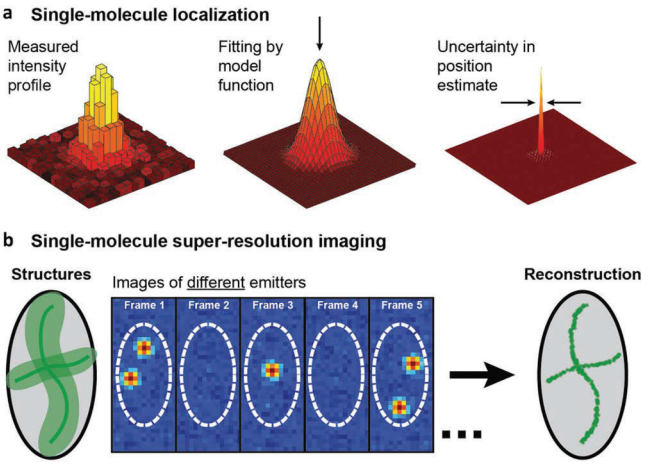

Fig. 1: Single molecule localization microscopy. a) The position of a single molecule can be determined to a much higher precision than the diffraction limit. **b)** Image formation in SMLM works by repeatedly determining the positions of a multitude of blinking molecules at high precision. The localizations are then combined computationally into a single super-resolved image (right). Adapted from [7].

An extension of SMLM to 3D is particularly of interest for objects that are thicker than the depth-of-field of a high-NA microscope, which is less than 1 µm. One of the ways to extend SMLM to 3D is to use point-spread-function engineering – a technique in which the microscope is modified optically by an additional optical element, namely, a phase mask, that changes the shape that a point source (e.g. a single molecule) generates on the camera, known as the point-spread-function (PSF). A molecule no longer appears as a single spot that becomes blurry upon defocus, like in a standard microscope, but it now has some distinct shape that efficiently encodes its depth, which can then be recovered algorithmically (Fig. 2a, b) [7], [8].

During the last decade, deep learning has been revolutionizing signal and image processing, and microscopy is no exception; it has enabled efficient image denoising, segmentation, multimodal registration and more [9]. Specifically, the ability of supervised deep learning methods to learn image reconstruction directly from optically encoded data relieves much of the effort that would traditionally be required for image decoding. In other words, the microscope designer needs to worry now mostly about injecting enough information in the image, i.e., encoding, rather than how that image will be decoded into the information of interest; in many cases, a neural net will take care of it.
Our group has been applying deep-learning to a variety of algorithmic challenges in SMLM and related topics. Notable recent examples include:

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- Localizing fields-of-view densely filled with fluorescent molecules, enabling faster image acquisition for SMLM [10]. This is done by training a neural net to recover high-resolution images, given low-resolution images comprising of randomly positioned emitters, either simulated or experimentally measured (Fig. 2c).
- Generating a super-resolution *video*, rather than a single image, directly from a blinking video, enabling live-cell super-resolution microscopy. This approach for x-y-t interpolation, termed DBlink [11], is implemented by relying on long-time correlations between frames, and training a recurrent neural network on relatively simple simulated localization maps.
- Localizing dense molecules in 3D in a PSF-engineered microscope, trained on simulated images of molecules in 3D, using an experimentally-calibrated image-formation model [12] (Fig. 2d). Furthermore – the neural net can be used to find the optimal encoding, i.e., the optimal phase mask pattern for dense 3D SMLM, which is the spiral mask shown in Fig. 2b (row 3), resembling a hummus plate. Additionally, in extremely dense cases, it is worthwhile to split the imaging path into two parallel channels, each encoded by its own phase mask, which can be jointly learned with the help of a neural net [13].
- Determining the diffusion type of single particles directly from their trajectories, using a neural net [14].
- Finding optimal spectrally-encoding phase masks for single emitter position + color estimation [15].

The techniques we develop are intended for use by a wide range of scientists. Therefore, an important concern is to lower the technical barrier for adoption. In the context of computational microscopy there are two main aspects to this hurdle: software, and hardware. On the software side – all algorithms we develop are published online, and we make an effort to make them user-friendly and accessible, e.g. by sharing them on platforms dedicated for this purpose [18], [19]. On the hardware front – one of the key barriers for adopting PSF engineering is the phase mask, which is a piece of dielectric material that normally requires sub-wavelength fabrication precision, which is expensive and cumbersome to achieve. To overcome this hurdle, we have developed a method to fabricate phase masks based on 3D printing combined with near-index matching, leading to fast and simple fabrication of phase masks with optical properties comparable to state-of-the-art masks, and in some cases even better-performing [20], [21].

Neural networks are likely to continue to play an important role in SMLM, both in image processing and in computational-imaging system design. New trends in neural networks are constantly finding their ways into SMLM, with notable recent examples from our group being transformer-encoders for direct image to DNA sequence mapping [22] and diffusion models for realistic super-resolution image generation for various purposes [23]. As neural nets are often used as “black boxes” with sometimes unpredictable behavior, extra care should be taken for their validation and performance evaluation in SMLM [24]. Ultimately, time will tell which neural network-based methods prove to be significantly valuable, useful, and robust enough to become the new state of the art.

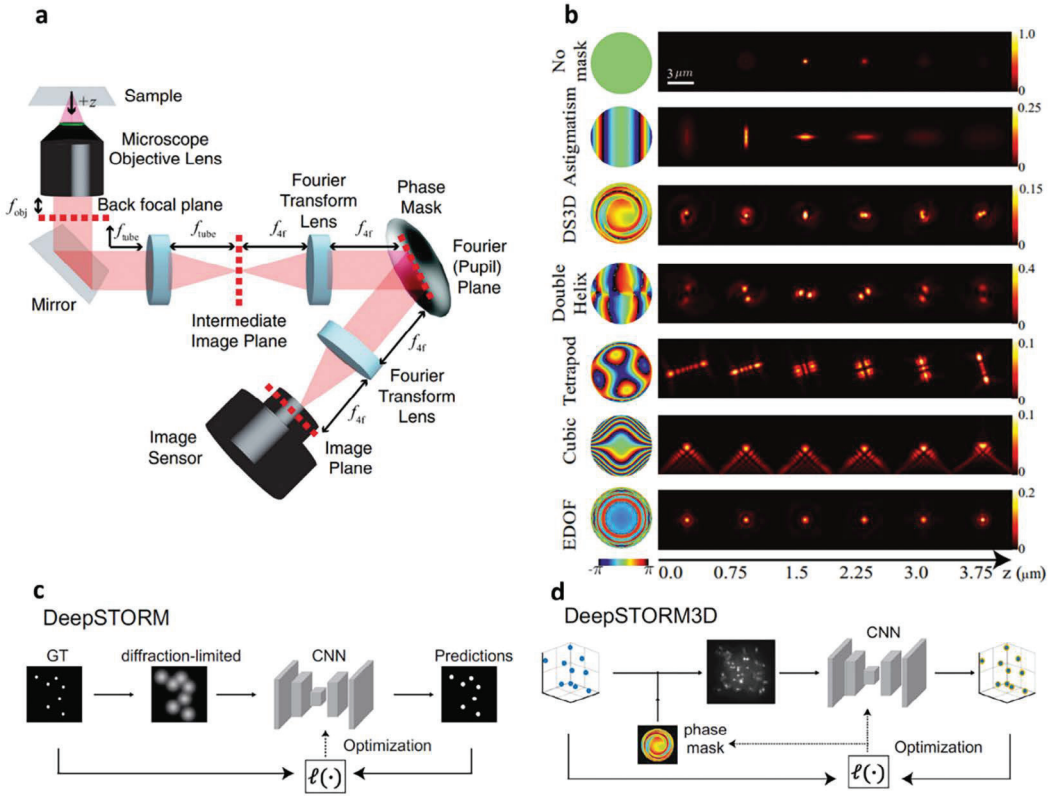

Fig. 2: Point-Spread Function (PSF) engineering and deep-learning based reconstruction. a) A microscope with an additional 4-F system with a phase mask in the Fourier plane for PSF modification (adapted from [16]). **b)** Example phase masks and their corresponding PSFs, as a function of defocus level, for 3D information encoding (rows 2-6) or for extended depth-of-field imaging (rows 7-8) (adapted from [17]). Example neural-net reconstruction concepts for **c)** 2D SMLM data [10] and **d)** 3D SMLM data [12], adapted from [9].

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