Parallel super-resolution imaging

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Massive parallelization of scanning-based super-resolution imaging allows fast imaging of large fields of view.

Ever since Ernst Abbe formulated the theory of diffraction in optical imaging, resolution has been thought to be restricted to approximately half the wavelength of light: the diffraction limit. Much work has been done to circumvent this limit; early work involved methods such as structured light illumination, first pioneered by Lukosz and Marchand and subsequently popularized by the late Gustafsson. More recent developments such as stimulated emission depletion (STED) microscopy, photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), reversible saturable optical fluorescence transitions (RESOLFT) and their variants have made great progress in bringing optical imaging to the 10-nm scale, but fast imaging of large fields of view using these techniques has been an elusive goal. In this issue, Chmyrov et al. present a novel parallelized RESOLFT approach that promises to eliminate the speed bottleneck in super-resolution imaging at these scales.

Techniques based on point spread–function engineering (such as RESOLFT and STED) typically generate an image by raster scanning a single point through a sample. After fluorophores are switched on within a diffraction-limited volume, RESOLFT works by switching off most of the fluorophores, allowing fluorescence from just one small region to dominate. Switching off is usually achieved using a ‘donut beam’, which has a finite intensity close to the center of the beam but a node at the very center where the light intensity drops to 0. The light from the donut beam is used to suppress the fluorescence from surrounding fluorophores, either by stimulated emission in the case of STED or by switching the fluorophore off photochemically in a typical implementation of RESOLFT, thus ensuring that the fluorophores at only the very center of the donut beam will be observed. The key to the RESOLFT principle is saturation: the idea that, with a sufficiently high light intensity, all the fluorophores in a particular region can be made not to fluoresce. Through the maintenance of a sufficiently high intensity in the donut beam, the remaining fluorescent region can be made arbitrarily small, limited only by the power that can be applied to the sample.

Conventional RESOLFT requires raster scanning of both the switch-on and the switch-off beams to generate a volumetric image. In comparison, techniques that can image the whole field of view in parallel, such as PALM and STORM, work by ensuring that only a statistically random subset of fluorophores are active simultaneously, such that the probability of having more than one fluorophore emitting within a diffraction-limited volume is negligible. Given these assumptions, the centroid of each fluorophore can be determined with nanometer precision, and the process can be repeated with many different subsets to create a high-resolution image.

In general, super-resolution imaging techniques are slow with small fields of view: the speed of raster-scanning techniques is limited by their sequential approach, and the speed of full-field techniques such as PALM and STORM is limited by the need for multiple exposures. Chmyrov et al. overcome the limitations of raster scanning with a new form of nanoscopy that greatly improves imaging speed by massively parallelizing the photochemical RESOLFT approach, resulting in imaging times of 400 ms for a 50 μm × 50 μm field of view or 2–4 s for a 100 μm × 120 μm field of view. Parallelized switch-off is achieved by projecting two orthogonal incoherent standing-wave patterns of high frequency on

Figure 1 | Parallelized RESOLFT imaging uses ~110,000 donuts to scan a sample. The different colored plots represent the intensities of excitation and emission light during imaging. The donut beam (blue) switches off fluorophores in the sample, whereas a uniform excitation beam (green) stimulates fluorescence (red) from switched-on fluorophore probes at the centers of the donuts.

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the sample. This generates a multitude of zero-intensity spots or ‘donuts’, each of which con-
fers super-resolution and is scanned to form a
super-resolved image (Fig. 1). Importantly, at
saturation, the cross-sections of the ~110,000
donuts are effectively circular, and no rotation
of the pattern is needed to ensure that resolu-
tion is isotropic.

Using this highly parallel super-resolution
setup, the researchers were able to image ker-
atin 19–rsEGFP(N205S) expressed in PtK2
cells; part of the cytoskeleton could then be
visualized with 80-nm resolution. In another
demonstration, the growth of neurites from a
neuron expressing the LifeAct-Dronpa-M159T
fusion protein was measured over time. Here
each super-resolution frame was measured in
2 s, allowing the growth to be monitored with
high spatial and temporal resolution.

An important goal in the methodological
development of this field is to achieve three-
dimensional (3D) super-resolution imaging
over a substantial volume and at video rates.
Chmyrov et al.17 do not quite achieve this: the
frame rates, despite showing major improve-
ment over those seen in previous efforts, are
still low, and super-resolution is demon-
strated in 2D rather than 3D, although the
authors report a commendable z resolution
of around 580 nm. Other methods such as
3D STORM10 and biplane fluorescence PALM19
have been shown to deliver sub-100-nm axial
resolution, but the stochastic nature of these
approaches results in substantially longer
imaging times.

At present, the authors note that it is the
camera and the state transition kinetics of the
fluorophores that limit the frame rate. Today
camera technology is advancing rapidly and is
likely to become a nonissue within a few years.
On the other hand, the development of more
efficient switchable fluorophores represents
the key bottleneck. Fortunately, many excel-
ent laboratories are actively on the hunt for
better fluorophores. Indeed, with the intense
interest in switchable fluorophores for not
just RESOLFT but also PALM, STORM, satu-
rated structured illumination microscopy18
and their derivatives, microscopists can look
forward to a future with a wide palette of dif-
ferent fluorophores optimized for nanoscopy.
When that happens, wide-field RESOLFT
imaging and its variants may well become a
commonplace substitute for wide-field fluo-
rescence imaging whenever high-resolution
images are required.

**Competing Financial Interests**
The authors declare no competing financial interests.

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**Enhanced dissection of the regulatory genome**

Matthew Slattery & Kevin P White

Methods for high-throughput and high-resolution dissection of enhancers in *Drosophila* are described by two independent groups.

Transcriptional cis-regulatory modules, or enhancers, are regions of DNA that integrate input from multiple transcription factors to direct precise spatial and temporal patterns of gene expression1. As such, enhancers are key components of the regulatory networks driving the developmental programs that shape meta-
zoan life. Despite their importance, enhancers
remain difficult to identify and characterize.
This difficulty arises in part because enhancer
regions are difficult to predict bioinformati-
cally and are often distal to the genes they
regulate, making enhancer bashing2 relatively
low throughput, but also because standard
reporter assays often remove enhancers from
their native context. In this issue of *Nature
Methods*, two groups describe experimental
approaches addressing problems with both
throughput2 and context dependence3 that
arise with enhancer characterization.

Genome-wide mapping of protein- and
histone-DNA interactions and DNA accessi-
bility has provided a starting point for large-
scale annotation of enhancers. However,
enhancer activities inferred on the basis of
transcription factor binding, chromatin
modifications and DNA accessibility can be
inaccurate and, when accurate, often lack
information regarding the enhancer’s tissue
or cell-type specificity. Therefore, inferences
based on genomic data must be followed up with functional assays, preferably assays
that provide information regarding tissue-
specific enhancer activity. Gisselbrecht et al.
begin to address this need with a method
termed enhancer-FACS-seq (eFS), which
increases the throughput of tissue-specific
enhancer characterization4.

The eFS strategy is based on a concept
underlying many traditional reporter-gene
assays: coincident expression of a reporter
gene and a tissue– or cell type–specific marker
indicates an enhancer is active in the given
subset of cells. However, eFS is highly paral-
lelized and allows for screening of hundreds of
enhancers at once (Fig. 1a). First, Gisselbrecht
et al.5 cloned thousands of candidate enhanc-
ers upstream of the GFP reporter gene. This
library of candidate enhancers was then used
to generate thousands of transgenic animals
(*Drosophila melanogaster*, in this case), each
carrying one genomically integrated enhancer-
GFP construct and expressing a tissue-specific
marker. As opposed to traditional reporter
assays, which are usually microscopy based,
eFS uses fluorescence activated cell sorting
(FACS) to capture cells expressing both the
tissue-specific marker and the GFP reporter
gene. Thus, GFP+ cells from various tissue
populations were collected via FACS. After
isolation of the DNA from this population of
cells, enhancer regions were PCR ampli-
fied and sequenced using next-generation
sequencing. With these enhancer ‘counts’ in
hand, enrichment in a given tissue relative to
the enhancer’s representation in the overall
embryo population provides a list of tissue-
specific, active enhancers (Fig. 1a).

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