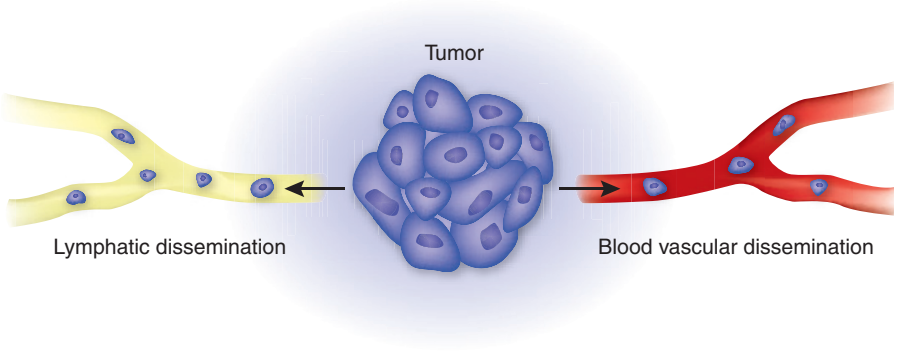

Figure 1 | As cells from a primary tumor are disseminated via blood or lymphatic vessels, methods to study angiogenesis and lymphangiogenesis will be valuable in understanding the basic biology behind the process of metastasis.

assay[1], whereas FGF-2 is usually far more potent than VEGF in most *in vitro* traditional endothelial cell monolayer assays. These differences may be due to matrix interactions.

The spheroid assay can also be modified to model lymphangiogenesis[1]. Human lymphatic endothelial cell spheroids revert to a blood endothelial cell phenotype when injected alone, but when lymphatic endothelial cells are mixed with human fibroblasts and implanted *in vivo*, human lymphatic networks form[1]. However, these grafted lymphatics do not appear to connect with host lymphatic vessels, suggesting that additional studies are needed to determine the proper conditions needed for lymphatic anastomosis.

In the other article, Bruyère *et al.* describe a new lymphangiogenesis assay[2], albeit *in vitro*. The assay uses fragments of the mouse thoracic duct implanted in collagen to model sprouting lymphatic vessels–similar to the murine aortic ring angiogenesis assay[7]. Within days of culture, the lymphatic rings show outgrowth of cells that organize into capillary structures enclosing a lumen[2]. The assay recapitulates the different steps of lymphangiogenesis and allows for the study of three-dimensional lymph vessels in an accessible, *in vitro* collagen gel matrix. Moreover, it allows for the study of lymphatic sprouting in the absence of blood vessels and of inflammation, which is difficult with *in vivo* assays.

Currently, there are very few lymphangiogenesis assays, and none model three-dimensional outgrowth *in vitro*. This new model[2] can be applied to the numerous transgenic mice already available. In addition, using computer-based image analysis developed by the authors, the assay is easily quantifiable[2].

The metastatic cascade via the vascular system involves a series of interrelated and coordinated steps[8,9]. Angiogenesis is essential for tumor progression, and anti-angiogenic strategies are a major focus of cancer research. The specific steps involved in tumor lymphangiogenesis and lymphatic metastasis are less clear[4], in part due to the lack of assays to study this process, but therapies aimed at blocking lymphatic vessel growth are being explored[4]. Both the spheroid assay[1] and the ring assay[2] recapitulate important steps in angiogenesis and/or lymphangiogenesis, including sprouting from existing vessels, cell proliferation, migration, maturation and lumen formation. These assays are likely to be extremely useful to analyze the effects of drugs directed against tumor neovascularization, as well as for studies on normal physiological development and tissue engineering.

1. Alajati, A. *et al. Nat. Methods* **5**, 439–445 (2008).
2. Bruyère, F. *et al. Nat. Methods* **5**, 431–437 (2008).
3. Folkman, J. *N. Engl. J. Med.* **285**, 1182–1186 (1971).
4. Zwaans, B.M. & Bielenberg, D.R. *Microvas.* Res. **74**, 145–158 (2007).
5. Dvorak, H.F. *et al. Lab. Invest.* **57**, 673–686 (1987).
6. Klagsbrun, M. Semin. *Cancer Biol.* **3**, 81–87 (1992).
7. Devy, L. *et al. FASEB J.* **16**, 147–154 (2002).
8. Fidler, I.J. *et al. Cancer J.* **4**, S58–S66 (1998).
9. Fidler, I.J. *et al. Cancer J.* **6**, S225–S236 (2000).

Super-resolution light microscopy goes live

Mats G L Gustafsson

Microscopic resolution far beyond the diffraction limit is possible by localizing single molecules individually. This approach has now been demonstrated on living cells.

The laws of diffraction set a fundamental limit to the spatial resolution of the conventional light microscope. The long-standing dream of doing away with this limit has been partly realized in recent years through various forms of super-resolution microscopy. Although impressive in resolution performance, these methods have until recently been restricted to dead, fixed specimens. A report in this issue of *Nature Methods* brings one powerful super-resolution technique into the realm of living, moving cells[1].

For a microscope that is attempting to form an image of an arbitrary sample, there is one main path to improve the resolution beyond the diffraction limit: by using spatially structured illumination light—in the form of either a scanned beam or a periodic pattern[2]—enhanced by nonlinear phenomena such as saturated transitions[3–5]. One highly developed nonlinear method, stimulated emission depletion microscopy (STED), for example, has achieved lateral resolution in the 20–30 nm regime on biological samples[3].

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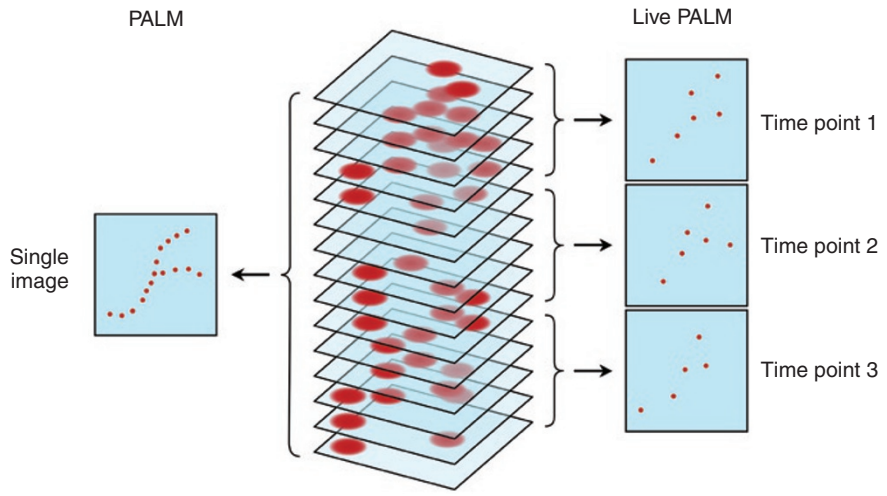

Figure 1 | The concept of live, time series PALM. A typical PALM dataset consists of a series of raw data images, in each of which only a few molecules are fluorescent (center). The position of each active molecule is determined precisely from the center of its blurred image; these position measurements are then gathered into a high-resolution reconstruction. In standard PALM the full dataset is used to produce a single reconstruction (left); this maximizes the quality if the sample is static but can result in motion blurring for moving samples. In live PALM, the data set is divided into a number of time points, which are reconstructed separately, allowing motion to be followed and decreasing the blurring—but also the available number of molecules—in each image (right).

About two years ago a different category of methods, known by acronyms like PALM, STORM and FPALM, leaped into prominence[6–8]. These do not attempt to form an image of an unknown, continuously varying dye density; instead they treat the sample explicitly as a collection of individual molecules. If a single molecule can be imaged alone, its position can be determined with great precision simply by determining the center of its blurred image on the camera. In photoactivated localization microcopy (PALM) and related techniques, a large number of fluorescent molecules are individually located this way, and a high-resolution image is produced from the list of estimated molecular positions.

The trick that makes it possible to image the molecules individually is to use photoactivatable labels: the labeling molecules start out in a dark state, and by illuminating with very weak activation light, a few molecules at a time are photoactivated to a fluorescent state, imaged and subsequently deactivated (photobleached). This process is repeated, often tens of thousands of times, to generate one output image. Because only a few molecules are fluorescent in any one input image, and these are spread out over a large field of view, their images are usually non-overlapping and can therefore be located precisely. This approach is capable of very high resolution, theoretically into the single-digit nanometer regime[3–5].

Many exciting results in modern microscopy come from imaging of processes in living cells and tissues, and super-resolution microscopy will be even more powerful if it can be applied to live samples. Unfortunately, many super-resolution methods have been quite time-consuming and therefore more suited to fixed, static preparations than to living specimens that would undergo internal changes while data was being acquired, causing blur. This situation has now started to change. A beam-scanning form of STED was recently demonstrated that could achieve 62-nm resolution at an impressive imaging rate of 28 frames per second, albeit over a small $1.8 \times 2.5\ \mu m^2$ area and with a signal level of only a few photons per pixel[9]. Tracking of photoactivated molecules has been used to study the movement of proteins in the plasma membrane of living cells, and images and videos have been formed from that type of data both with PALM[10] and with the very similar technique fluorescence PALM (FPALM)[11]. The diffusive motion was so rapid, however—diffusion coefficients around $0.1\ \mu m^2/s$ were measured—that substantial motion blurring would take place during the acquisition of any image that was to comprise enough located molecules to reflect the full structure. In this issue Shroff *et al.*[1] now show that it is possible, for a class of slower phenomena, to perform time-series PALM on living cells in a regime in which sample motion, density of sampled molecules and localization precision all come together to allow ~60-nm resolution in each time frame.

PALM as first published did not look like a promising candidate for dynamic imaging: to produce a single two-dimensional reconstruction it required 20,000–50,000 raw data images, which took 2–12 hours to acquire[6]. Shroff *et al.*[1] have since shortened the necessary acquisition time by several orders of magnitude, by increasing the camera frame rate and decreasing the number of raw data images used for each PALM image. Raising the frame rate was possible simply by increasing the excitation light intensity, which both increases the fluorescence emission rate and reduces the time required to photobleach each molecule, allowing more molecules to be localized per time unit. The decreased number of input images per reconstruction is necessary when attempting a time series: there are only a finite number of labeled molecules in the specimen, each of which is only localized once (PALM typically uses irreversibly activatable molecules), and in a time series these available molecules must be divided among the time points (**Fig. 1**).

In practice, input images are often acquired continuously for the duration of the time series and only later divided into time points. Thus there is a free choice of how many time points to use, which clearly involves a trade-off between temporal resolution and the quality of each PALM image. Shroff *et al.*[1] consider this tradeoff carefully, both in terms of noise (the randomness of a given molecule's being visible or not in a particular PALM image can be thought of as a kind of molecular shot noise) and in terms of the Nyquist sampling theorem: to resolve a structure with period d, sampling points (here equated to located molecules) cannot be separated by more than $d/2$.

In most of their data Shroff *et al.*[1] choose to assign about 25 seconds of raw data to each PALM image, with 4–20 such time points per series. On their preparations, this division of data provides sufficient sampling density for a target resolution of about 60 nm.

The target resolution sets the scale for how much motion can be tolerated during a time point without degrading the resolution. Sixty nanometers in 25 seconds is much slower speed than many cellular processes—vesicle transport, for instance, moves 400 times that fast—but there are phenomena for which this time scale is well-suited. Shroff *et al.*[1] studied the adhesion complexes in NIH 3T3 and CHO cells, which have dynamics on this timescale and are accessible by total internal reflection (TIRF) microscopy.

An important question about live PALM is whether the very large total exposure to excitation light, accumulated over the thousands of images, leads to prohibitive levels of photodamage. Shroff *et al.*[1] addressed this question experimentally and found no ill effects. This is not too surprising, as much of the photodamage in fluorescence microscopy is mediated by the excited fluorophore, and in PALM each fluorophore molecule is excited only for one bleaching lifetime, no more than it would be in conventional fluorescence microscopy. Photodamage is also limited by operating in the TIRF mode, where only a small part of the cell volume is exposed to light.

Although the time series presented in this article are very impressive in their molecular detail, they are relatively short, most comprising between 4 and 14 time points. This is a natural consequence of this form of PALM, in which fluorophore molecules are 'disposable': they are only activated once and never reused for any other time point. The finite number of available molecules cannot be distributed over more than a small number of time frames, if each frame is to contain enough molecules for proper sampling.

At first glance one might expect that the precise single-molecule localization information provided by PALM could be used for exquisitely detailed speckle microscopy: measuring organelle movement by tracking the motion of statistical variations in labeling density. Unfortunately that is not possible with the form of PALM that is described here because the images for different time points reflect disjoint subsets of fluorophores, which have uncorrelated random fluctuations.

Both limitations described in the two preceding paragraphs could be removed by using reversibly rather than irreversibly activatable fluorophores. If the total phototolerance remains similar, however, an increased number of activation cycles would lead to a decreased number of photons emitted by a molecule per cycle, which would decrease the localization precision.

Molecular localization microscopies like PALM are powerful tools for high-resolution imaging and are likely to have a substantial impact on cell biology. That this class of methods can be used successfully on dynamic living cells is very exciting news indeed.

1. Shroff, H., Galbraith, C.G., Galbraith, J.A. & Betzig, E. *Nat. Methods* **5**, 417–423 (2008).
2. Gustafsson, M.G.L. *et al. Biophys. J.*, published online 7 March 2008 (doi:10.1529/biophysj.107.120345).
3. Donnert, G. *et al. Proc. Natl. Acad. Sci. USA* **103**, 11440–11445 (2006).
4. Schwentker, M.A. *et al. Microsc. Res. Tech.* **70**, 269–280 (2007).
5. Gustafsson, M.G.L. *Proc. Natl. Acad. Sci. USA* **102**, 13081 (2005).
6. Betzig, E. *et al. Science* **313**, 1642–1645 (2006).
7. Hess, S.T. Girirajan, T.P.K. & Mason, M.D. *Biophys. J.* **91**, 4258–4272 (2006).
8. Rust, M.J., Bates, M. & Zhuang, X. *Nat. Methods* **3**, 793–795 (2006).
9. Westphal, V. *et al. Science*, published online 21 February 2008 (doi:10.1126/science.1154228).
10. Manley, S. *et al. Nat. Methods* **5**, 155–157 (2008).
11. Hess, S.T. *et al. Proc. Natl. Acad. Sci. USA* **104**, 17379–17375 (2007).