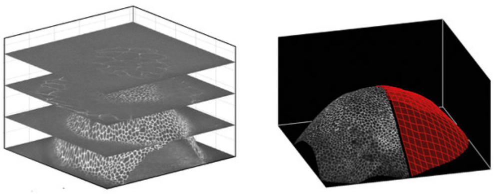
SMART ILLUMINATION FOR 3D-IMAGING OF BIOLOGICAL TISSUES

Loic LE GOFF*, Lorry MAZZELLA, Sofia CECCHINI, Nicolas LEVI-VALENSI, Marc ALLAIN, Anne SENTENAC, Frédéric GALLAND,
Aix Marseille Univ, CNRS, Centrale Med, Institut Fresnel, 13013, Marseille, France
*** legoff@fresnel.fr**



https://doi.org/10.1051/photon/202513436

Three dimensional imaging of living tissues with widefield fluorescence optical microscopy is limited by out-of-focus blur, light dose, and acquisition speed. We discuss recent camera-based strategies that use light more intelligently to improve resolution, sectioning and reduce toxicity when imaging thick biological structures.

The microscope as a scientific instrument for biological discovery emerged in the 17th century. When Robert Hooke examined a thin slice of cork under a microscope, he saw tiny compartments and named them 'cells', thereby helping to establish the idea that living tissues are made up of repeating units. For a long time afterwards, microscopes continued to reveal shapes — membranes, nuclei and filaments — without necessarily showing what those structures were doing.

Over the last two decades, the use of fluorescence has transformed biological imaging. Fluorescence is the process by which a molecule called fluorophore absorbs light at one wavelength, which excites its electronic state, and then de-excites by emitting light at a different wavelength. Using optical filters that only transmit the emitted light allows labelled structures to stand out with high contrast. This revolution was accelerated by the development of fluorescent proteins (FPs), which are genetically encoded tags that can be fused to proteins of interest inside living cells and organisms [1]. Through FPs, a wide variety of tools are available nowadays, including calcium or voltage reporters for neural activity, cytoskeletal markers for force and shape investigation, and stress or programmed cell death probes. Once the necessary genome edits have been introduced, entire organisms can be engineered to express these probes in selected tissues or throughout the body, enabling us to observe cellular behaviour in vivo rather than on an isolated glass slide.

Imaging cells within their host organism poses new technical challenges for the microscopist. It requires 3D imaging with high spatial and temporal resolution deep inside whole organisms, such as Drosophila embryos, zebrafish larvae or organoids. There are three practical hurdles to overcome. Firstly, out-of-focus fluorescence creates a blurred background that obscures details especially for deep observations. Secondly, building a 3D stack slice by slice increases the light dose, bleaching fluorophores and disrupting physiology. Thirdly, acquiring many planes is time-consuming; the dynamics we are interested in can exceed the capabilities of our scanners and cameras.

This article explores strategies that use structured and smart illumination to mitigate these constraints. The goal is simple: keep samples alive, capture events as they happen, and reveal structures that were previously hidden—by using light more intelligently.

3D-MICROSCOPY

Microscopes are inherently asymmetric instruments. Light propagates along a single, privileged direction — the optical axis. Imaging instruments naturally create contrasted images in the transverse (xy) plane,

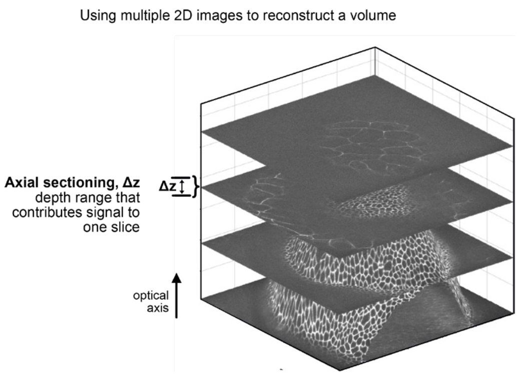


Figure 1. A three-dimensional structure is captured by acquiring a stack of 2D slices at successive positions along the optical (z) axis (4 of ~50 shown). Effective optical sectioning means each slice is sensitive only to a narrow axial thickness, Δz; if Δz is large, out-of-focus fluorescence from neighboring planes blurs the image.

while access to the third dimension (z) is indirect.

To create a 3D image with a conventional microscope, a stack of 2D images must be acquired while translating the sample along the optical axis. In principle, this method should work: each slice samples a different depth, and the stack encodes the volume (see Fig 1).

In practice, however, we encounter the sectioning problem. As the excitation light propagates along the optical axis, it also excites fluorophores above and below the focal plane that is optically conjugated to the camera. These fluorophores emit photons that are not part of the desired image plane; they travel back along the same axis and impinge on the camera as a blurred background. In link with this background, widefield microscopes discriminate objects poorly along the optical axis: a uniform fluorescent layer perpendicular to the optical axis cannot be localized, and two homogeneous layers at different depths are indistinguishable. The thicker the specimen, the stronger this unwanted background becomes.

This is why the confocal microscope, introduced as a solution for optical sectioning, has become such an important tool in biology.

CONFOCAL MICROSCOPY — RESTORING OPTICAL SECTIONING

A confocal microscope forms an image one point at a time (Fig. 2). A tightly focused excitation spot scans the sample. At the image plane conjugated to the focal plane, the in-focus fluorophores excited by the beam focus yield a small intense spot while the out-of-focus fluorophores, excited by the diverging beam, form a large low-intensity smear. By placing a pinhole strictly conjugated to the excitation focus spot, one ensures that most of the light emitted by the in-focus fluorophores is collected while most of the light of the out-of-focus fluorophores is discarded. Emission produced above or below the focus being largely blocked at the pinhole (Fig. 2), the microscope now records a slice with far less out-of-focus background. By repeating the scan at successive depths, a 3D stack is built with far better axial discrimination than the conventional -widefield- microscope.

While this introduces sectioning, it also comes at a price in the form of necessary trade-offs for 3D imaging. The pinhole rejects most of the emitted photons, many of which carry useful information, so the overall throughput is low. In order to maintain the signal, users often have to slow down the scan in order to accumulate more photons per pixel, which reduces the imaging temporal resolution; alternatively, they can open the pinhole, which sacrifices sectioning. Ultimately, the low usable signal per pixel (photon budget) forces the user to increase the excitation power. However, biological tissues can be very sensitive to light exposure. Excited fluorophores can generate reactive oxygen species (ROS) that can alter signalling or trigger cell death. In practice, the cumulative dose increases with the number of planes, dwell time and re-imaging frequency, meaning that 3D confocal imaging imposes stress that scales with volume and time.

In subsequent sections, we •••

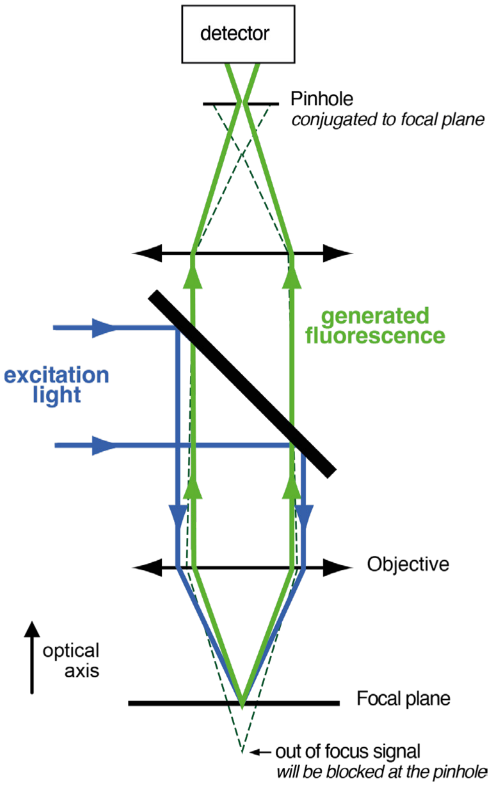


Figure 2. Confocal principle. The excitation light (blue) is focused to a spot that scans the sample. Fluorescence (green) passes through a small pinhole placed in a plane optically conjugate to the focal plane, so emission from above or below focus is blocked (dashed rays), producing a slice with much less out-of-focus background.

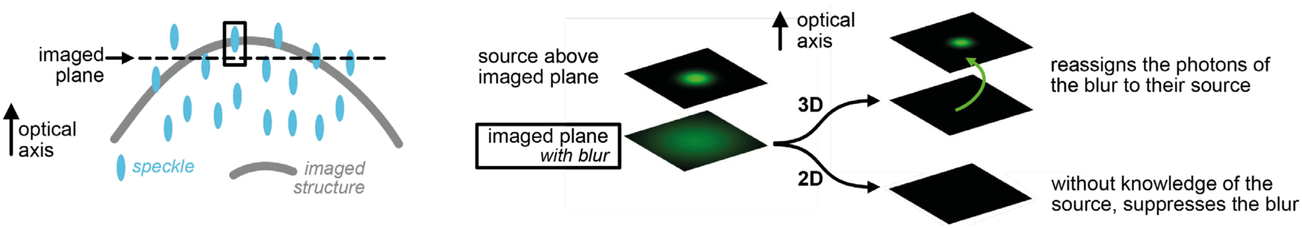
present complementary strategies that address these limits. Firstly, 3D Random Illumination Microscopy (3D-RIM) uses speckled excitation and variance-based reconstruction to recover super-resolved, optically sectioned volumes while reassigning photons to their correct depth. Secondly, an extended-depth-of-field (EDF) mode compresses the volume onto a single exposure to capture fast dynamics with a much lower readout burden. Lastly, smart illumination allows to reduce the light dose impinging on the sample by focusing the illumination patterns only where it is required.

3D-RANDOM ILLUMINATION MICROSCOPY

An alternative to point-scanning is Structured Illumination Microscopy (SIM, [2]): rather than scanning a focus, the whole field is illuminated with periodic patterns and several images are recorded with shifted phases and orientations. The patterned light down-modulates within the microscope's passband the information otherwise beyond the resolution limit. These images are combined numerically to yield a synthetic image with twice better resolution. In 3D-SIM, the pattern is also modulated along z, which improves axial as well as lateral resolution and provides sectioning for 3D imaging. The main drawback of 3D-SIM is that the reconstruction technique requires the knowledge of the illumination patterns inside the specimen. In embryos and other thick, heterogeneous tissues, aberrations and scattering distort the patterns in ways that are hard to measure or predict, so conventional 3D-SIM can produce artifacts or fail at depth.

In recent work [3], we proposed Random Illumination Microscopy (RIM) as an alternative structured illumination microscopy technique that avoids the knowledge of the excitation patterns. The sample is illuminated with a sequence of random speckle patterns and imaged with a camera at gentle intensity. Instead of using the patterns value at each point in the tissue, RIM reconstruction procedure exploits the statistics of the speckle ensemble. It extracts a super-resolved image from the variance of the multiple low-resolution speckled images using a variance matching inversion scheme. The RIM approach requires only the knowledge of the speckle short-range correlation and the observation point spread function. In addition to a two-fold resolution gain which has been mathematically demonstrated and experimentally confirmed [4,5], it provides numerical optical sectioning. The latter can be simply explained by first noting that the speckled illuminations form random bright grains throughout the sample volume. The fluorescence light emitted by the in-focus excited fluorophores yields sharp spots at the image plane, while the light emitted by the out-of-focus fluorophores yields large smears. When the speckled patterns are changed, the fluorescence coming from in-focus structures undergo large intensity fluctuations, whereas the blurred

Figure 3. 3D-RIM. Left: a 3D structure (grey) is imaged with a speckle, which appears as a random distribution of bright spots (blue). The imaged plane appears as a dashed line. We focus on a small region of interest (black box). Right: In that small box, the imaged structure is excited by a speckle grain above the imaged plane, thus contributing a blur in the imaged plane. A 2D approach (confocal or 2D RIM, bottom right) removes this blur because it has no knowledge of the source above the imaged plane. A 3D approach (top right) will reassign the out of focus photons to their source through a deconvolution.



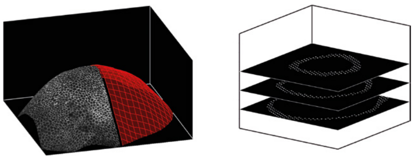


Figure 4. Smart-scanning. Sometimes, the signal of interest occupies a small fraction of the sample volume. Such is the case for example when cells are organized as a curve surface (left). We can then estimate a spatial model of the structure (computed surface in red), and concentrate illumination to a thin shell around this structure of interest (right).

contribution of the out-of-focus regions varies much less. As a result, the variance processing washes out the out-of-focus background.

In both the confocal and RIM procedures, the out-of-focus contributions are discarded physically or numerically: we end up using only the photons thought to originate from sources in the focal plane. This approach is justified if only one slice of the sample is imaged. On the other hand, if one wants to record a volume image of the sample, discarding the photons arriving from fluorophores above and below the focal plane is not a good idea as they carry information on the three-dimensional (3D) structure of the sample. This led us to develop a 3D version of RIM that uses the 3D nature of speckle and 3D data processing to exploit the information carried by these out-of-focus photons (see Fig. 3).

In *3D-RIM*, we sequentially image the volume of the sample while keeping the 3D speckle pattern fixed. Instead of moving the sample, axial scanning is carried out by a remote-focusing unit placed in the detection arm, such that the illumination stays registered and acts as a stable 3D probe. The structuring of the speckle along optical axis can then be used to improve sectioning. See the illustration in Fig. 3: when imaging a given plane, the fluorophores above or below this planes provide out-of-focus photons appearing as smears on the image. If we have knowledge of the 3D stack, however, we can attribute those out-of-focus photons to their source—effectively reassigning them to the proper plane. Thus there is no need to suppress the smears: we use them and put the photons back where they belong. Mathematically, this reassignment is performed through a deconvolution step before computing the 3D variance.

The consequences are that 3D-RIM keeps RIM's robustness in thick, aberrated tissues while adding stronger axial sectioning and better photon budget—fewer photons are thrown away by the variance step. In practice, the same level of detail can be obtained with lower illumination power, making the method gentler for live samples.

EXTENDED DEPTH OF FIELD

While faster than the confocal, structured illumination microscopies, including RIM, are slower than the widefield microscope as they require the acquisition of multiples images of the sample under different illuminations. In addition, thick 3D samples require many sequential slices to reconstruct a volume. Each newly acquired image also comes with a camera "dead time" linked to reading out of the information on the sensor. When biology is fast and the scientific question tolerates a projective view along z, we reasoned that compressing the whole volume into a single exposure becomes a powerful alternative. This can be done through extended depth imaging [6].

During each camera exposure, the focal plane is swept rapidly through the specimen using a tunable element (for example, an electrically tunable lens). The sensor therefore integrates fluorescence from the full axial range, yielding one extended-depth frame. Repeating this with a set of different speckle illuminations provides the data for variance-based reconstruction, which removes the characteristic blurred background that normally mars extended depth images. We could thus retrieve super-resolved details in the projection.

Compared with acquiring a full 3D stack with standard RIM, EDF-RIM reduces the number of readouts and can be about ten times faster, while maintaining lateral resolution. The price to pay is that axial information is compressed into •••

the projection; when full volumetric reconstruction is required, 3D-RIM remains the method of choice.

We could validate the instrument on biologically relevant thick samples, including mouse intestine tissue, Drosophila embryos, and human cultured cells, illustrating both robustness and live-imaging compatibility.

SMART SCANNING

Camera-based 3D imaging offers speed and high resolution, providing gentler, more distributed illumination than point-scanning confocal systems. However, even with parallel acquisition, very sensitive tissues can be damaged. Background fluorescence also poses a fundamental limitation: the photon noise associated with background can overwhelm the speckle-based variations used for super-resolved reconstruction, undermining the capacity for super-resolution.

To overcome these issues, we have developed a smart microscope that focuses illumination only where necessary, placing the imaging process under algorithmic control and enabling structures of interest to be targeted progressively [7]. Real-time analysis learns from the measurements already acquired and chooses where to probe next, so the illumination converges toward relevant features such as curved embryonic surfaces (Fig. 4). When we have a strong prior knowledge of the structure of interest -as is the case with surfaces, for example - it may take as few as three steps: performing a very sparse pre-scan; estimating a spatial model of the object (in this case, a surface); and scanning the surface. In more complex situations, a longer iterative approach may be required to converge. In our initial study, we focused on cell sheets, which are curved 3D surfaces commonly found in embryos, but the concept is general.

In the future, the detection could also be trained through examples, using machine-learning and artificial intelligence approaches. Temporal continuity is also crucial: information from one time point could inform the next, allowing the acquisition to concentrate on changes and maximize new information per irradiation photon.

The benefits of smart scanning are first in the reduction of the light dose. Because the structures of interest often occupy only a small fraction of the total volume, the illuminated voxels – and thus the total light dose impinging on the sample– can be reduced by up to two orders of magnitude. Lower dose means less phototoxicity and less bleaching, enabling much longer observations of living tissues. At the same time, restricting illumination to the informative regions reduces background and its associated photon noise, which improves reconstruction quality. A last practical side effect is strong data compression: by measuring less but measuring smarter, we store and process only what matters.

PERSPECTIVES

Using light more intelligently can improve contrast, resolution, speed, and sample health in 3D imaging. By placing excitation photons only where they are informative, we extend what live fluorescence microscopy can do without harming the specimen.

As a perspective, crossmodality is the natural next step. We can pair 3D-RIM or EDF-RIM with adaptive illumination patterns that target regions of interest in real time. Cross modality with point scanning microscopy (non-linear point scanning is still the way to go at really great depth), and light sheet microscopy will also be explored.

In all cases, the aim is to maximize information per photon, minimize dose, and go faster when imaging thick tissues.

ACKNOWLEDGEMENTS

These results are the outcome of a nation-wide interdisciplinary collaboration. We are especially grateful to Nicolas Bertaux, Simon Labouesse, Thomas Mangeat, Jérôme Idier, Benoît Rogez, Faris Abouakil, Marie-Anne Burcklen, Hervé Rigneault, Guillaume Giroussens.
This project is funded by the "France 2030" investment plan managed by the French National Research Agency (ANR-21-ESRE-0002), and from Excellence Initiative of Aix-Marseille University—A*MIDEX, as well as Agence Nationale de la Recherche ANR-22-CE13-0039, ANR-22-CE42-0010, ANR-24-CE13-2163.

REFERENCES

[1] A. M. Gest, A. Z. Sahan, Y. Zhong, W. Lin, S. Mehta and J. Zhang, Chemical Reviews **124(22)**, 12573 (2024)

[2] M. G. Gustafsson, Journal of microscopy **198(2)**, 82 (2000)

[3] T. Mangeat, L. Mazzella, B. Rogez, G. Giroussens, C. Bernard, P. Vargas, M. Allain, S. Labouesse, J. Idier, L. LeGoff and A. Sentenac, (3D-RIM) (2025). BioRxiv. DOI: 10.1101/2025.08.06.668879

[4] S. Labouesse, J. Idier, M. Allain, G. Giroussens, T. Mangeat and A. Sentenac, Physical Review A **109(3)**, 033525 (2024)

[5] T. Mangeat, S. Labouesse, M. Allain, A. Negash, E. Martin, A. Guénolé and A. Sentenac, Cell Reports Methods **1(1)**, (2021)

[6]L. Mazzella, T. Mangeat, G. Giroussens, B. Rogez, H. Li, J. Creff, M. Saadaoui, C. Martins, R. Bouzignac, S. Labouesse, J. Idier, F. Galland, M. Allain, A. Sentenac and L. Legoff light: Science & applications **13(1)**, 285 (2024)

[7] F. Abouakil, H. Meng, M. A. Burcklen, H. Rigneault, F. Galland and L. LeGoff, light: Science & applications **10(1)**, 210 (2021)

COMPLETE SOLUTIONS FOR QUANTUM APPLICATIONS

How Menlo Systems' Optical Frequency Combs and Ultrastable Lasers Enable the Second Quantum Revolution

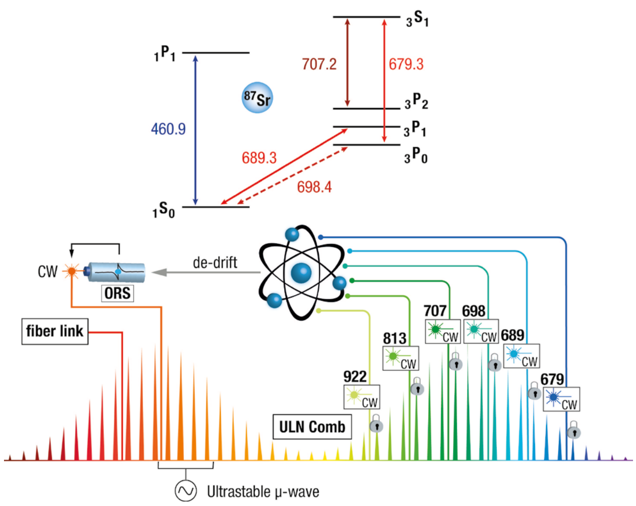
Quantum technologies are driving a new era of innovation by exploiting quantum physics for real-world applications. Their ability to measure, compute, and communicate with unprecedented precision makes them essential for tackling today's most complex scientific and technological challenges. Particularly impactful is quantum sensing, harnessing fragile quantum states of atoms, ions, or photons as ultra-sensitive probes of their environment, enabling breakthroughs in navigation, geodesy, and tests of fundamental physics.

To realize these capabilities, researchers need tools that combine absolute stability, coherence, and reproducibility. From quantum simulators, communication, and computing to atomic sensors and metrology, precisely stabilized light is needed to manipulate atoms and ions, define timing and phase relationships, and establish coherent links between quantum systems.

This is where photonics comes in: optical frequency combs are mode-locked pulsed lasers whose spectrum consists of thousands of evenly spaced, phase-coherent lines. When locked to an ultrastable reference, the combs inherit this stability and transfer spectral purity to other wavelengths at the 10^{-18} level, enabling precise probing of atomic transitions.

Menlo Systems has served the optical community with high-end optical frequency combs for nearly 25 years and provides a fully integrated, commercial solution for quantum and timekeeping applications: the FC1500-Quantum. The system combines an optical reference and an ultrastable frequency comb with several continuous-wave (CW) lasers for atom cooling, repumping, and addressing narrow clock transitions in atoms or ions. Low phase noise of the comb-disciplined lasers ensures the phase coherence required for precise state manipulation. Laser light can be fiber-delivered to the physics package, simplifying integration with vacuum systems and optical setups. Acting both as a reference for all lasers and as the spectral bridge linking optical and microwave domains, frequency combs are the heart of modern quantum laboratories [1].

A showcase example of the FC1500-Quantum in action is Fermilab's MAGIS-100 (Matter-wave Atomic Gradiometer Interferometric Sensor) [2] project, which aims to explore fundamental physics through atom interferometry over a 100-meter vertical baseline. Here, clouds of ultracold strontium atoms are launched in free fall while laser beams, stabilized by the FC1500-Quantum, act as beam splitters and mirrors for matter waves. Several watts of sub-hertz light at 698.4 nm are collimated along the baseline. The system's ultrastable frequency and phase coherence are critical for this quantum sensor, which could probe ultralight dark matter, test the equivalence principle and pave the way for future gravitational wave detectors.



▲ The atomic transitions in Strontium (Sr) atoms with the ultra-narrow clock transition at 698.4 nm (upper part). A commercial FC1500-Quantum system for optical clock applications contains an ultrastable laser transferring its spectral purity onto an optical frequency comb and all other lasers which are also locked to the comb (lower part).

Another example of frequency-comb-aided quantum-enabled precision is found in optical atomic clocks, which are poised to redefine the SI second. Unlike the current standard cesium clocks, which are based on transitions at roughly 9 GHz, optical clocks leverage the stability and accuracy of ultra-narrow atomic transitions in the optical range, typically hundreds of THz. Frequency combs form the core of such systems and act as clockworks, counting trillions of oscillations. These state-of-the-art clocks allow for a precision of 10^{-18} [3], corresponding to an error of 1 s in 15 billion years. With collaborations like MAGIS-100 and the Boulder Atomic Clock Optical Network (BACON) [4], Menlo Systems is pushing the boundaries of quantum sensing and metrology. ●

REFERENCES

[1] M. Giunta et. Al.: Nat. Photonics 14 (2020), 44–49

[2] Mahiro Abe *et al.* : Quantum Sci. Technol. 6 (2021) 044003

[3] E. Oelker *et al.*: Nat. Photonics 13 (2019), 714 – 719

[4] Boulder Atomic Clock Optical Network (BACON) Collaboration: Nature 591 (2021) 564–569