The smart and gentle microscope

Nico Scherf & Jan Huisken

New instruments are needed to realize the potential of quantitative and systematic imaging of living samples. But what would such a microscope look like?

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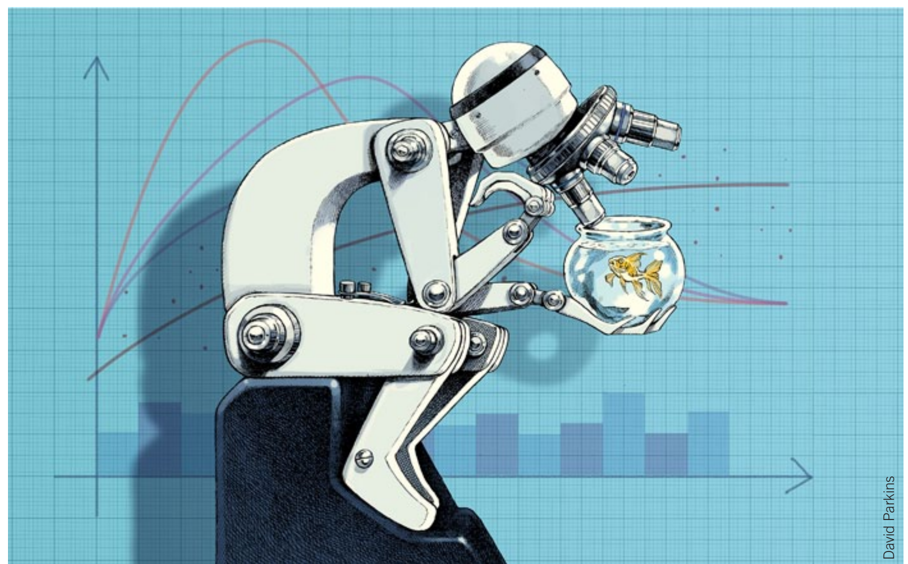
Let us consider a simple question for the moment: why does a modern commercial microscope body still look the same as a microscope body from a few hundred years ago? Apart from the fact that the fundamentals of optics haven't changed, the very purpose of a microscope is still the same; it is a tool that enables us to observe with our eyes the microscopic world, the fabric of life. Indeed, the microscope's physical shape and dimensions are essentially unchanged. It still has eyepieces and operates through visible light; and it stands on a desk, awaiting the scientist who sits down in front of it and observes the sample. Is this the microscope design that will help us achieve the type of quantitative and systematic imaging of living samples required to transform our understanding of biology? Will it enable us to carry out the type of large-scale imaging studies we envisage for the future? In the following Commentary, we ask what such a microscope of the future should look like. We argue it is time to leave historical constraints behind and create novel instruments that feature more than just good optics, to reveal the invisible world.



The thinking microscope.

New design principles

In biology, visual observation has always been a crucial step toward understanding, but the complexity of living matter and the impact of our measurements limit what we can achieve by traditional microscopy. First, multicellular life is complex and dynamic, formed by an orchestrated interplay of cells in a hierarchical process across different scales in space and time[1,2]. This complexity is beyond what we can visually comprehend by simply looking through a microscope. A thorough understanding of multicellular life requires more than traditional visual inspection; it requires quantitative measurements and statistics on a larger ensemble to judge the variability and significance of the findings.

Second, although microscopy should ideally be performed noninvasively in intact, living specimens, traditional instrumentation usually dictates that we mount (or worse still section) and image the samples under quite unnatural conditions. Many optical measurements require high light intensities inside the specimen, far beyond that which the sample would ever be exposed to under normal daylight conditions. Thus, our insights come at the price of drastic interventions and the results obtained by such invasive methods may be questionable. Therefore, to understand the fundamental processes of life, we need to image and analyze large numbers of living samples for as long as needed under the most physiological conditions we can provide and with the optimal spatial and temporal resolution. Achieving these goals in parallel demands a change in how we design and build microscopes—it requires our microscopes to become smart and gentle.

Beginning with the publication of Hooke's *Micrographia*[3] in 1665, microscopy has been the fundamental technique to unravel the mystery behind multicellular life. The microscope revolutionized our view on the inner workings of biological matter. It has become a key technique in diverse areas, such as developmental biology, cell biology and bioengineering. Fluorescent labeling techniques now allow us to visualize specific cellular structures

Nico Scherf and Jan Huisken are at the Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany; Nico Scherf is also affiliated with the Institute for Medical Informatics and Biometry, Faculty of Medicine Carl Gustav Carus, TU Dresden, Dresden, Germany.

Box 1 Imaging tradeoffs

The photon budget per specimen is limited, so the requirements for an ideal data set are diametrically opposed: sample health, temporal resolution, spatial resolution and quality of the images (or, more precisely, signal-to-noise ratio; see **Fig. 1**). It is impossible to optimize all of them at the same time; improving one will compromise another.

Ideally, one would like to image as fast as possible for a long period of time. But excited fluorophores typically give rise to oxidant species; the resulting phototoxic effects increase with light intensity and photon energy[23]. Biological tissues suffer damage far earlier than they undergo photobleaching. In particular ultraviolet and infrared light can cause severe structural damage and heating. Thus, a higher number of illumination cycles, and higher light intensities compromise the sample.

Aside from sample health, improving one particular technical aspect of imaging will almost always have deleterious effects as well. Higher temporal resolution leads to shorter exposure times and, consequently, reduced image quality. Additionally, high frame rates can be achieved only in small regions of the camera chip, compromising spatial resolution. Improved spatial resolution comes at the price of decreased photon counts per pixel; for example, doubling the resolution yields eight times fewer fluorophores per imaged volume, thus eight times more light or time is needed to get the same signal per pixel (at the cost of sample health or temporal resolution).

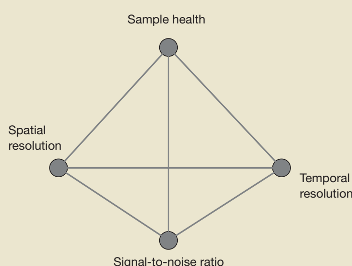


Figure 1 The key requirements for an ideal imaging data set are interdependent, resulting in tradeoffs between sample health, temporal resolution, spatial resolution and signal-to-noise ratio.

(e.g., the nucleus or the cytoskeleton) or functional states of cells (e.g., calcium release in activated neurons)[4]. The ability to optically section and study intact specimens with confocal and multiphoton techniques has opened windows into the structure of living organisms, which are three-dimensional without exception. Furthermore, the development of super-resolution optical microscopy (whose inventors were awarded the Nobel Prize in Chemistry in 2014) has enabled us to visualize the intracellular world beyond the diffraction-limited view of classic light microscopy[5]. Our deepening knowledge of the fundamental architecture of biological matter critically relies on the continued progress and evolution of microscopy techniques.

Understanding the full complexity of life's organization requires systematic *in toto* imaging approaches[6]. In addition to high spatial and temporal resolution in intact organisms, we also need long-term observation and, most importantly, large sample counts to address nature's intrinsic diversity. But imaging hundreds of intact specimens is beyond what we can achieve with existing techniques. The rather low speed of acquisition and the considerable amount of photodamage (bleaching and phototoxicity) limit the applicability of most fluorescence microscopy techniques. Particularly for cell and developmental biology, the most corrupting aspect of imaging is phototoxicity[7].

We need to revise our traditional techniques and remember that keeping the sample happy should be the hallmark of *in vivo* imaging, ideally without detriment to the sample's health or physiological development; simply speaking: the patient needs to survive. But requirements for improved resolution and image quality are typically antagonistic to the ideal conditions for the sample (**Box 1** and **Fig. 1**). So there is a tradeoff to be made and, at least for systematic developmental biology, we should change emphasis from pushing the image resolution even further to improving sample health. Steps in this direction were made a decade ago with the development of light-sheet microscopy[8] (**Box 2** and **Fig. 2**). It is now crucial to extend the idea of minimally invasive imaging. Scientists need to be able to image faster and with a softer touch; then will we discover new aspects of biology, unobscured by artifacts of the measurement.

With an ideal, minimally invasive microscope, the health of the biological specimen is no longer the speed-limiting factor; the sample can be imaged as fast as technically possible, for as long as needed. However, this freedom would create another technical challenge and bottleneck: the large size of the acquired data. Modern light-sheet techniques generate an enormous volume of images (many terabytes per day) that need to be transferred, stored and analyzed, putting pressure on the limits of even state-of-the-art infrastructures. Big image data have limited most experiments to a single sample and prevented systematic analysis of large ensembles. Although dedicated hardware and software might alleviate the problem[9], treating acquisition and processing as mutually independent is no longer a viable solution. By acquiring blindly and processing later, we obtain image data that are vastly larger than the specific information sought from the sample (e.g., dynamic tracks of cell movements and average fluorescent level per cell). Instead, we need a flexible, smart acquisition concept that delivers information-rich data with minimal overhead. As an intrinsic benefit the sample would be less exposed through more discriminatory data acquisition. Achieving this will require feedback between imaging and data processing to record only what is necessary. Ideally this should happen right at the source—in the microscope, at the camera.

Implementing this vision requires a paradigm shift in microscopy. It will no longer be the scientist in front of a microscope that observes each image—so most traditional design principles will be obsolete. Now is the time to leave the path of designing microscopes for manual operation and to abandon historical constraints. It is not enough to merely add a digital camera or digital controls to a conventional microscope; instead, acquisition and processing must be tightly connected so that the microscope adapts to the sample, acquires only what is essential to the particular question and leaves the rest untouched. Why do we still take rectangular images of organically shaped biological specimens?

Intelligent feedback between control, acquisition and processing is a basic requirement of such an approach. It provides the flexibility to optimally use all available resources and to get the most information from each experiment within our time constraints. Closed-loop feedbacks have proven useful for imaging in research and in industry for different purposes including autofocus, automation of screening experiments[10] or quality control. Adaptive optics yielded impressive improvements in image quality in astronomy and more recently in biology[11], particularly for deep tissue imaging. However, for systematic *in vivo* microscopy it is not insufficient quality, but the sheer amount of image data, that poses the primary challenge. Thus, the idea

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of adaptive acquisition has to be extended to selectively collect only data of interest in the first place, tailored to the particular question at hand.

The microscope of the future also needs to be gentle; it should be built around the sample. More natural ways of positioning the sample are required, which ultimately dispense with any form of embedding or mounting. A gentle microscope should allow the specimen to grow, swim or fly in its natural environment. It should be the microscope that adapts to the sample and not the sample that is fitted to the microscope. Being more physiological will allow us to obtain more and longer measurements of each specimen without destroying it, so the number of animals required per study can be reduced (referred to in animal science as reduction and refinement). Ideally, the microscope facilitates integration with complementary modalities (such as optical projection tomography[12]) to obtain as much information as possible per sample[13]. Ultimately, at the end of a time-lapse experiment, one could euthanize the sample animal and use invasive techniques, such as sectioning, super resolution or even electron microscopy, to complement the *in vivo* data with higher resolution data for the final time point.

From here to there

Although all this is a vision for the future, we sketch below a few ideas as to how we may take the first steps to reach our goal, and we outline some of the challenges that lie ahead, *en route* demonstrating that we are getting ever closer to overcoming some of the limitations discussed. Building the ideal platform will be an incremental process; the microscope cannot be fully smart and omniscient from the first day onward. Its implementation will be a process of learning, with each step increasing the degree of abstraction, feedback and flexibility.

The optical setup. The fundamental requirements for volumetric imaging of dynamic biological processes are fast and gentle acquisition. The microscope's hardware therefore needs to be based on a flexible and efficient microscopy concept that offers speed and low photoxicity. Light-sheet microscopy (**Box 2**) may be ideally suited with its selective illumination and fast widefield detection. Additionally, the optical system should be able to optimally shape the illumination beam and to adapt the detection readout to the structure of the sample under study. These improvements will yield increased resolution, penetration and contrast, and they will reduce readout times and overall phototoxicity.

Recent efforts have mainly focused on designing thinner, more penetrating (and oftentimes more harmful) light sheets by using multiphoton and beam-scanning excitation[14]. Nevertheless, in this context adaptive optical elements (e.g., deformable mirrors, spatial light modulators, tunable lenses) have become available, which could now be used to shape (and reduce) light exposure. Even so, it may turn out that adapting the light to the sample will require extensive probing in several iterations that simply takes too much time and effectively offers no benefit. In addition to the passive imaging of the specimens, in the next generation of smart microscopes, the optical setup could also incorporate targeted photomanipulation of the sample, such as with smart optogenetics[15].

Data acquisition of an individual sample. The microscope should reduce the data stream right at the source—the camera. For a single, initially unknown specimen, the smart microscope automatically recognizes the region(s) of interest (ROI); pixels holding no relevant information are not read out. Furthermore, large, scattering objects are rotated when illumination and detection can penetrate to the ROI only in certain geometries. Instead of taking a series of predefined angles (traditional multi-view imaging), the smart microscope automatically selects the angle(s) from which it gets the optimal view of the ROI. During repetitive (time-lapse) imaging of the same sample, information from previous time points could be used to adapt the ROI accordingly. Certain regions are imaged more frequently than others, depending on the sample geometry and how much the sample changes. In doing so, the acquisition speed is further increased and redundant data omitted, resulting in adaptively sampled, multi-resolution data sets (akin to online geographic maps).

Although it sounds straightforward, adaptive acquisition is challenging to implement in practice. Naturally, when building scientific optical instruments we depend on off-the-shelf components, which are mostly beyond our influence (e.g., objectives, cameras). A close collaboration with manufacturers and vendors will be necessary to push the development of dedicated components, such as cameras that offer readout of free-form ROIs beyond the usual rectangles. The emerging adaptive (open source) hardware platforms may offer interesting alternatives for some parts of our new microscopes.

Fast online processing of images provides a further source for optimization. During acquisition, data of insufficient quality should be rejected and the current ROI reimaged with optimized parameters. This approach avoids

Box 2 Light-sheet microscopy

Light-sheet microscopy (**Fig. 2**) offers a solution to the fundamental problems of acquisition speed and photodamage in traditional three-dimensional fluorescence microscopy (confocal or multiphoton). Intrinsic optical sectioning is achieved by illuminating the sample with a sheet of light and generating fluorescence only in the plane of interest, which is then imaged with a fast camera. This concept provides several advantages.

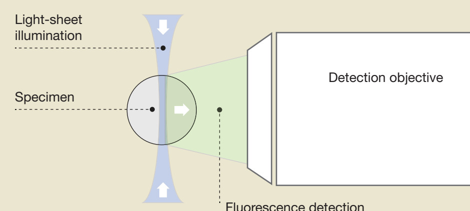

Figure 2 The principal components of a light-sheet microscope.

Minimal phototoxicity. Only the imaged focal plane is illuminated during each acquisition. When acquiring a stack of images of, for example 100 planes, each plane is exposed only once. In a confocal microscope the entire volume is exposed every time; that is, the last plane has already been exposed 99 times before it gets imaged. The reduced light exposure in light-sheet microscopy facilitates a higher number of acquisitions; that is, faster temporal sampling and longer total observation times.

Fast image acquisition. Widefield detection captures the entire image at once, collecting photons in every pixel simultaneously. Millisecond exposure times and frame rates of >100 frames per second are typical. This collection efficiency is crucial for imaging fast biological processes and is a prerequisite for multi-view acquisition.

Multi-view imaging. In some light-sheet microscopes, the sample can be rotated and imaged from different sides[24]. Fusing the obtained data sets increases the information content and resolution of the final image, in particular when deconvolving the data at the same time[25].

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recording experimental data that turn out to be unusable during subsequent processing (when it is too late to acquire better data). This will also force us to rethink the way image analysis is done in biology. Instead of using only singular results (e.g., the 'best' segmentation result), it is important to have at minimum a confidence measure for each result or ideally even a distribution over possible solutions. To later integrate these results into a statistical model, we will need precise information about the technical variability introduced by our methods.

Systematic data acquisition of many samples. Acquiring data for a large ensemble of samples bears the risk of recording and handling lots of redundant data and renders large-scale experiments impossible. The microscope should be able to integrate previously obtained data into a unifying reference model[16], selectively acquire the missing information and strengthen the statistics in subsequent experiments.

The obvious challenge here is to decide which data to discard and which to keep. This should be done in a principled way, using methods to optimize the experimental design by focusing attention on uncertain parts in the current model[17,18]. Here, the microscope does not necessarily have to decide autonomously; feedback by the scientist could be taken into account, at least as a first step. In this way, a biological process could be resolved at different relevant scales in its entirety over many samples; the result being a quantitative, statistical model of the process[19]. Constructing a reference model (digital atlas) of complex biological specimens is still a challenge requiring advanced methods from computer vision, differential geometry and spatiotemporal statistics. It will be important to foster the exchange of ideas with other disciplines, as there are many related problems in medical imaging, geoscience and astronomy.

Sample treatment. For understanding the dynamic processes of life, the fixation of the sample as the first item in a protocol is a step backwards. So what is needed are less restrictive embedding techniques[20] or, ideally, no embedding at all. So far, it has been accepted that we need to mount our samples to hold them in place and to position them for three-dimensional imaging. But some organisms are too sensitive to be embedded or manipulated.

Of course, if the sample is free to move, the acquisition speed needs to be sufficiently high to resolve sample movement. Rapid volumetric imaging techniques have recently been developed[21,22] providing a solution to ultimately dispense with any form of embedding and immobilization of the sample. Thus, we could image freely swimming larvae of aquatic species in parallel under identical (and natural) external conditions.

Conclusions

The smart and gentle microscope is a general concept and a desirable vision for the future rather than one concrete design made manifest. Different applications will require a certain amount of customization, as each specimen has its own peculiarities. Some are more opaque, some move very fast, some are very sensitive. Successfully addressing any one of the individual tasks will be a considerable improvement to *in vivo* microscopy, but it is their incorporation into a unifying framework that is likely to change the way we can do quantitative imaging. Although some steps have already been taken for niche applications, the next step now is to bring them together for a specific, well-defined example; ideally a specimen with a geometrically simple shape, such as a spherical zebrafish embryo or an ellipsoidal fly embryo. Then, we can further extend and generalize this imaging toolbox, step by step, to more complex examples. Ultimately, the goal is to have a general platform for a wide range of applications that automatically detects the object and acts accordingly. Modularity in designing the system will likely be the key to success.

The microscope of the future will look different from a traditional setup; indeed, no two implementations will look identical. Integrating knowledge from biology, optics, engineering and computer science, it will be more than an optimized magnifying glass. It will be a modular and adaptive imaging system built around a physiological environment designed for the specimen under study. Keeping the sample happy will allow us to observe relevant biological phenomena and not artifacts of our measurement procedures. A smart measurement will prevent us from drowning in data and allow us to capture all relevant aspects of a biological process. Optimally using the resources available, we will no longer be limited to only a few samples, but finally be able to thoroughly study nature's variability. The smart and gentle microscope will be an entirely new tool to address the current challenges in quantitative biology and facilitate new kinds of *in vivo* experiments, which crucially are systematic and noninvasive.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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