Chapter 1

Instrumentation for Live-Cell Imaging and Main Formats

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Abstract

Unlike immunofluorescence confocal microscopy of fixed samples or microscopic surface analysis in material sciences that both involve largely indestructible samples, life-cell imaging focuses on live cells. Imaging live specimen is by definition minimally invasive imaging, and photon efficiency is the primordial concern, even before issues of spatial, temporal or, spectral resolution, of acquisition speed and image contrast come in. Beyond alerting the reader that good live-cell images are often not the crisp showcase images that you know from the front page, this chapter is concerned with providing a fresh look on one of the routine instruments in modern biological research. Irrespective of whether you are a young researcher setting up your own lab or a senior investigator choosing equipment for a new project, at some stage you will most likely face decision making on what (fluorescence) imaging set-up to buy. In as much as this choice is about a long-lived and often relatively costly piece of equipment and, more importantly, impacts on your future experimental program, this choice can be a tricky one. It involves considering a multitude of parameters, some of which are discussed here.

Key words: Fluorescence, live-cell imaging, microscopy, instrumentation.

1. Introduction

Fluorescence microscopy has evolved from an add-on contrast mode of the laboratory light microscope to a puzzling multitude of formats probing different aspects of molecular fluorophores. Classically requiring nothing else but a bright white light source for wide-field illumination (often termed a "burner"), a set of fluorophore-specific filters housed and purchased as a preassembled "cube," and an imaging detector ("camera"), the rapid technological evolution of scientific instruments has involved virtually all elements of the fluorescence microscope.

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Fundamental choices for the user concern the illumination source, where arc-lamps are increasingly being replaced by lasers or high-power light-emitting diodes (LEDs) as discrete-wave-band illumination devices, pulsed or continuous-wave excitation, point scanning vs. whole-field excitation, filter-based or dispersion-based fluorescence band selection, integrating vs. photon-counting detectors, multi-channel or spectral detection, intensity of lifetime detection, to name only a few. To these options concerning instrumentation add those coming from the rapid progress in the synthesis and generation of molecular fluorophores, photolabile caged compounds, fluorescent and photoswitchable proteins, and photoactivated ion channels that often call for specific add-ons and imaging modalities. Moreover, the detection of intrinsic signals (autofluorescence, scattered light, higher harmonic generation) offers interesting alternatives to conventional fluorescence imaging depending on the introduction of exogenous probes.

For a novice, it might appear difficult to navigate through this diversity of instrumentation, formats, and probes and to have an educated choice among the variety of equipment or software available. This chapter, without attempting to be complete, is meant to provide the groundwork for choosing and evaluating instrumentation for live-cell imaging. Emphasis is on principles and constraints imposed by the different techniques rather than on a detailed discussion of specific equipment.

1.1. Further Reading and Web Resources

It is beyond the scope of this introduction to provide a detailed discussion of the ever increasing number of different formats of fluorescence microscopy. Fluorescence microscopy in its many variants is a standard theme in undergraduate and graduate courses, and a number of excellent reviews and textbooks are devoted to this subject; see below for a selection. To these, we have added the online resources provided by the different scientific societies as well as companies. We also alert the reader to the many excellent hands-on training courses that are held each summer and which – at least for the more prestigious ones – combine excellent theoretical training with the possibility to get your hands on the most recent pieces of equipment and thus provide valuable information before decision making about which piece of equipment to get for your own lab. Other important sources of first-hand information are the numerous cost-free optics and photonics journals.

1.2. Selected Fluorescence Textbooks

Although by no means complete, these recent (re-)editions of classic books provide an in-depth coverage of many aspects of fluorescence microscopy techniques, with a specific emphasis on biological and live-cell imaging.

1. Lakowicz, J. R., Principles of Fluorescence Spectroscopy, Springer, Heidelberg, New York, 3rd edition, 2006
2. Pawley, J. B. (ed.), Handbook of Confocal Microscopy, Springer, Heidelberg, New York, 3rd edition, 2006
3. Goldman, R. D. and Spector, D. L. (eds.) Live Cell Imaging – A Laboratory Manual. CSHL Press, Cold Spring Habor, 2005
4. Imaging in Neuroscience and Development – A Laboratory Manual. CSHL Press, Cold-Spring Habor, 2005

1.3. Web-Based Resources

Almost all microscope suppliers now offer free online tutorials that cover many aspects of microscopy: resolution, contrast generation, microscopic optics, and basics of fluorescence microscopy. They also point the reader toward related courses (often organized in partnership with the companies) and review articles.

1. Olympus Microscopy resource center: http://www.olympusmicro.com/primer/java/index.html
2. Zeiss Microscopy, http://www.zeiss.com/, and then link to "Technical Information"
3. Nikon Microscopy, http://www.microscopyu.com/tutorials/, and, specifically on confocal microscopy, http://www.microscopyu.com/articles/confocal/
4. Leica Microsystems, http://www.leica-microsystems.com/website, then link to "Leica Scientific Forum"
5. Molecular Expressions Images from the Microscope, National High Magnetic Field Laboratory (NHMFL), Tallahassee, http://micro.magnet.fsu.edu/

1.4. Courses

There is an ever-increasing number of courses that permit both theoretical training and hands-on experience. Here are some of the better known ones:

1. Marine Biological Laboratory (MBL), Woods Hole, http://www.mbl.edu/education/
2. Cold Spring Habor Laboratory, Cold Spring Habor, http://meetings.cshl.edu/courses.html
3. NIH Bio-trac courses, Bethesda, http://www.biotrac.com/pages/courses.html
4. Live-Cell Microscopy Course, UBC, Vancouver, http://www.3dcourse.ubc.ca
5. Quantitative Fluorescence Microscopy Course, Mount Desert Island Biological Laboratory (MDIBL), http://www.cbi.pitt.edu/qfm/index.html

6. Marine Biological Association, Plymouth, The Microelectrode Techniques for Cell Physiology, http://www.mba.ac.uk/events.php
7. European Molecular Biology Organization, Practical Courses, EMBO Course on Light Microscopy in Living Cells, Heidelberg, http://www.mba.ac.uk/events.php

A more comprehensive list is found by linking to http://www.olympusfluoview.com/resources/courses.html

1.5. Free Photonics Journals

Another valuable source of information for beginning as well as confirmed microscopists is freely available monthly journals. A particularity of the photonics market, there are quite a few of them. Although often in close (sometimes all-too-close) proximity with advertiser and manufacturer opinion, these publications are a showcase of recent developments in optics, microscopy, and biophotonics. They provide up-to-date information on new equipment, notable technical achievements, and provide an excellent overview of trade fairs and meetings, both to come (which is good if you are to chose components for your microscope), or in the form of brief synopses, reviewing recent trends. While not replacing the academic literature, they certainly broaden your horizon and keep you connected to the often rapidly evolving technology at no extra cost.

1. Photonics Spectra
2. Europhotonics
3. Biophotonics International, all three from Photonics Media, Laurin Publishing, http://www.photonics.com
4. BioTechniques, from Informa Healthcare, http://www.biotechniques.com
5. Optics & Laser Europe, IOP Publishing, http://www.optics.org
6. Photonik international (*German*) from AT-Fachverlag, http://www.photonik.de
7. Photoniques, de la Société Française d'Optique, http://www.photoniques.com

1.6. Keep Your Cells Alive

When imaging live samples, the first question arising is how to use the limited photon budget without compromising sample viability. Careful controls should be made to develop a quantitative notion of how the used intensities affect the biological process under study. The result is often surprising because photodamage starts gradually before obvious signs occur. For example, two studies investigating how ample was two-photon photodamage when imaging intracellular free calcium ($[Ca^{2+}]_i$) (1, 2) found that the slope and kinetics of neuronal calcium signals were attenuated much earlier than electrophysiological signs of

photodamage occurred. Thus, before starting your imaging, begin by thinking how you can

- optimize the photon yield (i.e., the number of collected signal photons vs. photons injected into the sample),
- avoid excitation wavelengths at which the sample absorption (and hence heating and photodamage) is strong,
- minimize the applied dye concentration,
- avoid producing crisp showcase images but aim for live cells,
- repeat the same experiment using different excitation intensities and using different emission bands,
- choose the imaging format that excels in your specific application.

This last point is crucial because it involves a choice of equipment and may lead you to the conclusion that the ideal experiment is impossible with existing material and can only be realized through an external collaboration. **Figure 1.1** provides a scheme (3) that helps rationalizing this decision making. Although oversimplistic, this scheme is useful because it points at the limitations of particular techniques and brings up useful parameters that should go into your consideration, e.g., background rejection, optical sectioning, imaging speed, or penetration depth (to only name a few).

1.7. Do You Really Need a Microscope?

Free yourself from the acquired wisdom that the choice is among the diverse upright and inverted scopes offered by the "big four," Zeiss, Leica, Nikon, and Olympus, and that the decision is merely a question of preference, compatibility with existing equipment, or the best commercial offer. Instead, ask yourself the following questions:

- Do I really need a microscope?
- Do I need eyepieces, bulky microscope bodies, inaccessible and unchangeable intermediate optics, and a limited flexibility governed by the elements to select from the suppliers' catalogue?
- You probably already have a good routine microscope for cell culture, patch-clamping, or checking immunofluorescence labeling before going to your facility's confocal or routine imaging. But do you need to combine different imaging formats as expensive (and often sub-optimal) add-ons on the same instrument?
- Or can you do better, with dedicated, small, and inexpensive set-ups?

A number of companies now offer modular solutions that allow you to configure the microscope the best way according to your needs. These solutions provide an interesting alternative

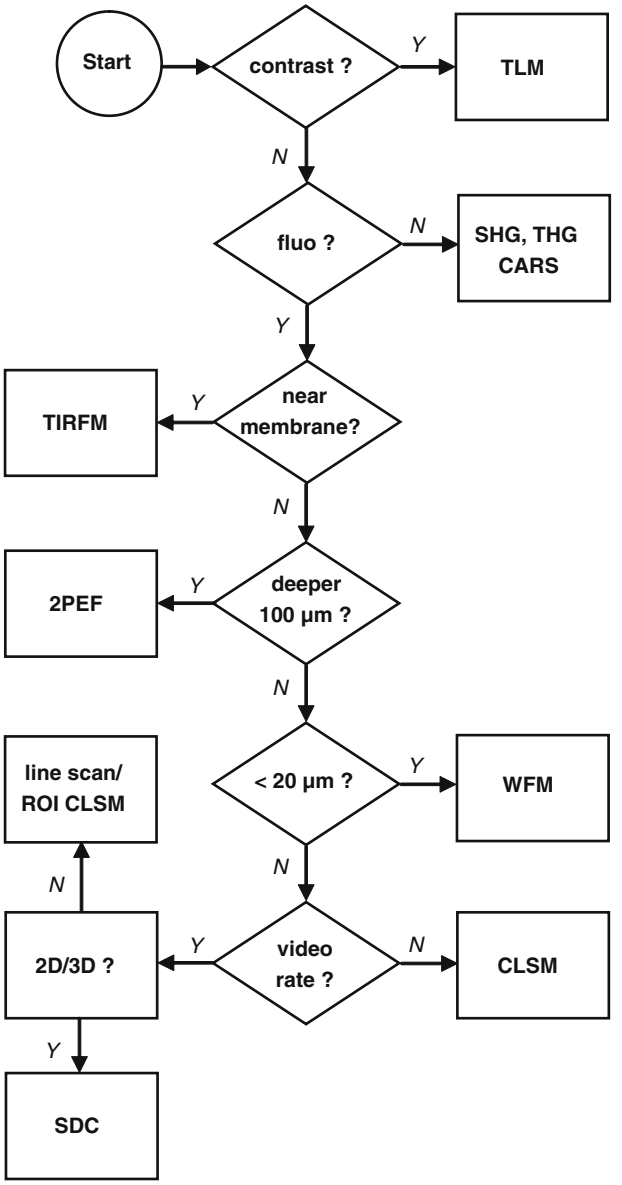

Fig. 1.1. *Abbreviations:* Y/N – yes/no; TLM – transmitted-light microscopies; SHG, THG – second (third) harmonic generation; CARS – coherent anti-Stokes Raman scattering; TIRFM – total internal reflection fluorescence microscopy; WFM – wide-field microscopy; 2PEF – two-photon excitation fluorescence; CLSM –confocal laser scanning microscopy; ROI – region of interest; SDC – spinning-disk confocal.

to the often somewhat finicky custom microscopes assembled from optical bench systems and also provide a means to generate hybrids between commercial microscope components and custom optomechanics. All of these approaches have in common that they offer full control of what you put in your microscope and allow replacing specific components that become limiting in

a given application. We later discuss two examples of such custom microscopes built in our own laboratory.

Below, some of such modular microscopes the author is currently aware of are listed.

1. Olympus BXFM series (part of the BX51/BX61 upright microscope series). Most components of this upright microscope are sold individually, permitting to build your own structures.
2. Zeiss Axio Scope 1.Vario, a more recent but similar, component-based upright and highly modular microscope originally designed for the material sciences, http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/8FE44E3197A08FEBC125742E005BD1E1.
3. Already somewhat more reductionist is the SliceScope from Scientifica. It is a commercial minimally stripped down microscope body (without eyepieces) for combined electrophysiology and DIC/fluorescence imaging, http://www.scientifica.uk.com.
4. A similar system, equipped with Dodt contrast, is available from Siskiyou, http://www.siskiyou.com/imaging_system_mrk200-infrared-fluorescence.shtml.
5. TILL Photonics, offers a highly modular automated microscope that even goes below a bench or a robotic sample handler. Here the approach is rather to rethink the microscope body and to evolve in the direction of screening-by-imaging, http://www.till-photonics.com/Products/imic.php. With their now selling YANUS-4 laser scan head driven by all-digital smartmove boards, they equally offer a building block for constructing your own confocal or two-photon laser scanning microscope.
6. Somewhat more integrated, Prairie Technologies' Ultima is a confocal attachment with (optionally) two sets of galvanotmetric mirrors or acousto-optic deflectors for combined imaging and uncaging, http://www.prairie-technologies.com/ultima.htm.
7. Becker & Hickl has a confocal scan head (DCS-120) and detector modules for both intensity and fluorescence lifetime measurements, http://www.becker-hickl.de/.
8. Even further toward DIY, the opto-mecanical component supplier Linos (formerly Spinder und Hoyer) has its classical microbench, a 40 by 40 mm cage and rail system with components (eyepieces, revolver, lenses, C-mounts, apertures, ...) ready to build a microscope from scratch, http://www.linos.com/pages/home/shop-mechanik/banksysteme/mikrobank/.

Also exists in a 20 × 20-mm nanobench version. Unfortunately, the future of the even larger macrobench (150 × 150 mm) is with a question mark.

9. AHF (http://ahf.de) has the missing part for the microbench: a 45° holder for a 1-mm 25 × 63-mm (Zeiss or Olympus) standard dichroic mirror.
10. Interestingly, Thorlabs offers a fair number of cage system components that are largely compatible with the Microbench, thus considerably enlarging the choice of optical components, http://www.thorlabs.com/navigation.cfm?Guide_ID=2002.

2. Instrumentation

2.1. Understanding the Building Blocks of the Laboratory Microscope

It is quite instructive to forget what you know about laboratory microscopes for a moment. Modern fluorescence microscopes are highly modular and can be thought of as a box with lots of arms sticking in and out (**Fig. 1.2**). Excitation arms can be different channels of epi-illumination, a laser injected through a side port for total internal fluorescence or photoswitching, a spinning-disc confocal attachment, or a pulsed UV-lamp for flash-photolysis.

For a given excitation channel i and fluorophore j, excitation is fully described in terms of the source spectral emission $S(\lambda)$, transmission of the excitation filter $EX(\lambda)$, and the reflectivity diachroic beamsplitter $(1–BS(\lambda))$, which, for a give excitation channel, are multiplied along the excitation optical path:

$$\mathrm{ex}_i(\lambda) = \mathrm{S}(\lambda) \cdot \mathrm{EX}_i(\lambda) \cdot (1 - \mathrm{BS}(\lambda)) \quad [1]$$

combined with the sample molar extinction ε_j and absorbance spectrum $E_j(\lambda)$ and integrated over λ to give an excitation spectral function:

$$\xi_{ij} = \int d\lambda \, \mathrm{ex}_i(\lambda) \cdot E_j(\lambda) \cdot \varepsilon_j. \quad [2]$$

On the emission site, one proceeds analogously for each detection arm k by multiplying the fluorophore quantum yield and emission spectrum, spectral transmission of the dichroic and emission filters, and the detector spectral sensitivity:

$$\mathrm{em}_k(\lambda) = \mathrm{BS}(\lambda) \cdot \mathrm{EM}_k(\lambda) \cdot \mathrm{D}(\lambda). \quad [3]$$

Upon integration,

$$\xi'_{jk} = \int d\lambda\phi \cdot F_j(\lambda) \cdot \mathrm{em_k}(\lambda) \quad [4]$$

Finally, the product of eqs. [**2**] and [**1.4**],

$$\xi_{ijk} \equiv \xi_{ij}\xi'_{jk} \qquad [5]$$

measures the signal of one mole/l of fluorophore *j* viewed through channel *k* upon excitation in channel *i*, and similarly for all permutations *ijk*.

Optimizing the photon yield now consists in maximizing the detection spectral function, while balancing the excitation spectral function with that of other fluorophores present in the sample.

This balancing can be achieved not only by choosing appropriate filters but also by considering the source and detector spectra and the microscope intermediate optics and objective lens. Their transmission can conveniently be combined with that of the beamsplitter, thereby accounting for the double passage of excitation and emission light through the microscope.

The following list provides a quick overview of the principal choices available and briefly discusses their respective advantages and disadvantages with respect to the scheme shown in **Fig. 1.2**.

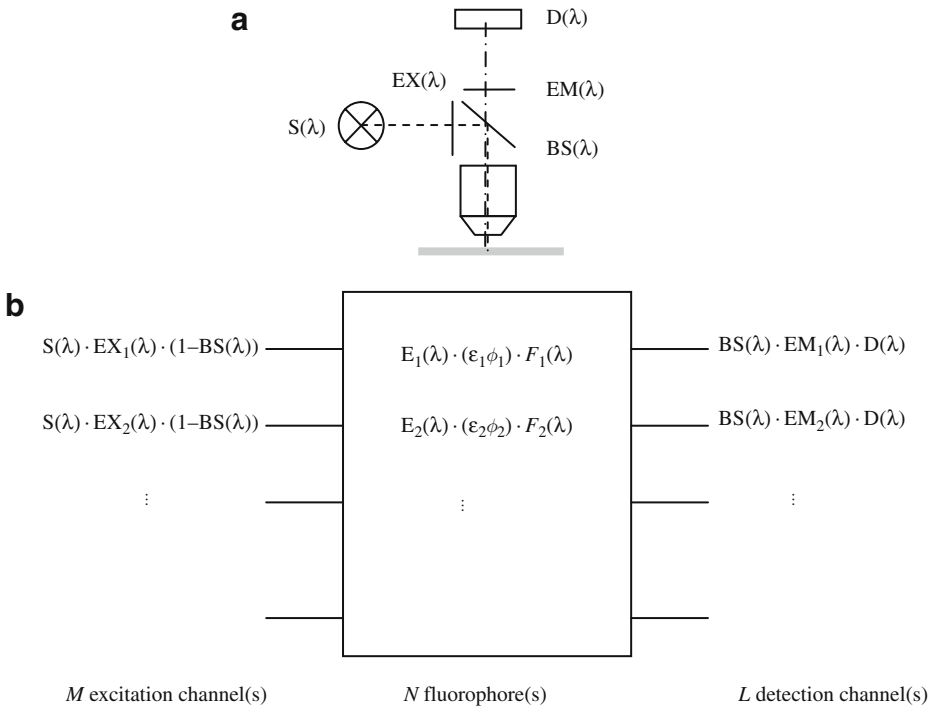

Fig. 1.2. (**a**) Schematic respresentation of an upright epifluorescence microscope. S – source; EX – excitation filter; BS – diachroic beamsplitter; EM – emission filter; D – detector. See main text for details. (**b**) Box plot of the different excitation and emission channels along with their spectral throughput, obtained by multiplying instrument and fluorophore spectral properties along the excitation and detection arms. See main text for details.

Light sources: the choice is no longer only between Hg- and Xe-burners but involves a large variety of sources including arc-lamp based monochromators, lasers (gas, diode-pumped solid state, DPSS), laser diodes, white light and color LEDs, and photonic crystal fibers for supercontinuum generation. These vary in

- wavelength, tuning range, bandwidth and power,
- luminous density,
- noise and long-term stability,
- cost,
- coherence, polarization,
- pulsed vs. continous-wave (CV) operation.

Excitation filters: typically, band-pass filters are used to narrow down the source spectrum and select specific wavelength bands, clean up laser lines, attenuate illumination intensity, or select a polarization direction. Main choices are between hard- and soft-coated interference filters as well as holographic notch filters. Important selection criteria include the following:

- their in-band transmission and off-band rejection (optical density),
- bandwidth or line-width,
- tolerance to high illuminating intensities (burnout, hole-burning).

  A similar reasoning must be made for the *dichroic mirror* that, for the more common long-pass, separates excitation and emission light by reflecting the excitation light onto the sample and transmitting the collected fluorescence. Chief parameters to consider in addition to center wavelength and steepness are as follows:

- Spectral holes: many dichroics perform nicely close to the transition wavelength, but display large variations and spectral holes at remote wavelength. "Extended range" ("XR") dichroics can be a solution, as are the new hard-coated filters that typically outperform the older soft coatings.
- Transmitted excitation light. Although often not a problem, low-light applications can suffer from the residual transmitted light that can be orders of magnitude more intense than the collected fluorescence. Stacking dichroics or matched long-pass filters can be an answer.
- Finally, particularly for multi-color scanning microscopies, the angle-dependence of the cut-on of the dichroic can result in surprising chromatic changes depending on whether paraxial regions or the periphery of the field-of-view is imaged. Typically, the cut-on wavelength changes as

$$\lambda(\theta) = \lambda_0\sqrt{1 - (sin\theta/n_{\text{eff}})^2}, \qquad [6]$$

For angles θ increasing beyond 45°, n_{eff} is determined by the dichroic of the order of 1.7–1.86 for most of the coatings, but varies for *p*- or *s*-polarized light so that their average must typically be considered for the collected fluorescence.

The *microscope intermediate optical path* (tube lens, scan lens, epi-illuminator, projection lenses in the case of compound magnifiers) and the *objective* are often not considered in detail, until unanticipated losses occur. For example, both UV and near-IR transmission become a problem, e.g., in fura-2 Ca^{2+} imaging, flash photolysis, or photoactivation, as well as two- or three-photon microscopy using far-IR excitation.

- UV transmission. With the increasing demand for highly corrected objectives (and tube lenses), high refractive index glasses and optical cement are often used chromatic corrections that limit UV transmission. Also, with microelectronics getting smaller and smaller, the formerly used UV-light inspection techniques often fail because of resolution limitations. Thus, dedicated UV-transmitting optics is getting rarer and rarer.
- Another often overlooked aspect is limiting intermediate apertures (filter cubes, lenses, irises) that reduce the collected light fraction of scattered fluorescence photons in two-photon microscopy. While scattered photons are usually rejected in fluorescence imaging (because they are not assigned to the pixel of their origin and hence blur the image) they constitute useful signal in 2PEF microscopy that seeks to optimize the light collection. Thus, keeping the product of $d\sin(\theta_{\text{eff}})$ constant is of crucial importance for not using light. Here, d is the size of the imaged spot and θ_{eff} is the half-angle of the effective numerical aperture.

Much of the same reasoning is true for choosing and optimizing detectors. Know your microscope is the rule toward getting optimal results.

2.2. The Performance Triangle

Irrespective of all these parameters, you can only distribute your collected photons once. Thus, whenever you take an image of a live cell, you take from the budget of photons that your sample emits before irreversibly undergoing photodamage and photodestruction. If you invest them into higher spatial resolution, higher temporal resolution or larger image contrast (signal-to-noise ratio) is your choice. And it is often a difficult one.

Resolution vs. magnification. On most available microscopes, resolution is diffraction-limited. Thus, the smallest distance that two objects can be close by and still be detected as two is defined by the numerical aperture of the objective, according to Abbe's

law. Importantly, the detector spatial resolution (i.e., the pixel size in an imaging detector, or the scan angle for a laser-scanning microscope) must be adapted, according to Nyquist's sampling theorem: two picture elements (pixels) per resel (resolution element), $0.61\lambda/(\mathrm{NA})$. Smaller pixels do not enhance resolution but increase photobleaching by void oversampling.

Superresolution, i.e., imaging beyond the diffraction limit has attracted wide interest over the last years and extensive reviews have been published. By narrowing down the fluorescence excitation volume (through stimulated emission depletion, STED, or structured illumination and image reconstruction methods) or by sequentially imaging individual fluorophores and reconstructing the image from the sum projection (STORM, PALM, and its variants) high spatial frequency information can be obtained. But again, superresolution translates into sampling at higher spatial frequencies, so that at a constant photo budget, either the dye concentration in the sample must be increased or the field-of-view must be reduced. Also, some super-resolution techniques require sample pre-bleaching, photoactivation, or the STED beam in addition to the conventional excitation light. It is safe to say that for many of these exciting developments, a critical evaluation of the photodamage resulting in the live samples still has to be done.

Image contrast comes from the number of meaningful signal photons over the unwanted background in a given fluorescence detection channel. Thus, spectral considerations directly come into play. If contrast is generated by splitting up the signal in many different spectral channels, then each of these channels will contribute to the noise and the signal-to-noise ratio will inevitably drop. Therefore, "multi-"spectral imaging is generally preferable over "hyper-"spectral imaging and a small number of detection channels followed by spectral unmixing outperforms full-blown spectral images (4, 5). Similarly, contrast in a given spectral bin can of course be increased by cranking the laser power up, but this again increases photobleaching and tears from the available photon budget. Therefore, it is useful to keep the "performance triangle" of fluorescence microscopy (*see* **Fig. 1.3)** in mind, any improvement in one of the image parameters comes at the expense of the others.

2.3. Additional Considerations

- *Long-term observation* of live samples critically relies on constant observation conditions, both in terms of the instrument (i.e., maintaining the focal stability and minimizing thermal drift) and of the biological sample (control of physiological temperature, ambient CO_2, and humidity levels). Due to condensation, placing the entire microscope in an incubator is not preferred, but many suppliers offer small-on stage or plexiglass-box incubators that can be fitted to many routine microscopes.

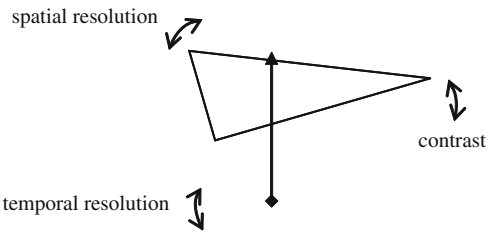

Fig. 1.3. The performance triangle. Distributing a constant photon budget into any of these three imaging parameters inevitably reduces the other two.

- Deciding between a *motorized vs. manual set-up* is not only a question of price and convenience. Many microscopists like the manual and thus more direct focus and stage control, e.g., when placing recording electrodes or local perfusion systems under visual control. On the other hand, many motorized systems now come with a fairly well-developed software, allowing the user to generate look-up tables of stage positions, objective *z*-positions or even automated follow software that keeps patch pipettes already placed above the sample plane in the field-of-view whilst searching for the cell to record from.
- *High-throughput microscopy* is increasingly becoming an option through the ongoing integration of machine vision, robotics, microfluidics, and automated analysis software. Several commercial systems are available, albeit at high cost.
- *Shared set-ups vs. single-user set-ups.* Perform a realistic evaluation of beamtime: which fraction of your experiment time is effectively being used for imaging? Which part is devoted to preparing and installing the sample? Which steps could equally be performed elsewhere to free precious beamtime? Most laboratory microscopes are under-used, but multi-user set-ups require clear shared responsibilities, agreed-on standard operation protocols, and an efficient communication among users. Otherwise, the gain in instrument use will easily be eaten up by problem solving and conflicts of unhappy experimenters.
- *Multi-functionality vs. dedicated set-up.* Beyond budgetary and space constraints, this often is a question of the type of experiments you have in mind. All too complicated microscopes are expensive, error-prone, and rarely all contrast modes are being used in the same experiment. Thus, "small is beautiful" is a guideline that more often than not gives good returns, particularly when set-ups are shared or even open to external users. Trained personnel and regular maintenance will make all the difference.

- *Optimal optical performance* in a given imaging format often involves custom equipment or add-ons to commercial microscopes. Typically being fairly labor-intensive, alignment sensitive, and often run with custom software that resembles beta-versions, these "expert systems" are less user-friendly but outperform standard equipment. Many recent imaging formats, including light-sheet based illumination, HILO (6, 7), but also versatile STED or 2PEF microscopes, still require building your own setup. At the same time, instrument development and building is typically longer than hoped for, so that the researcher has to evaluate the need for quick results against instrument performance.
- *Laser safety* is obviously a concern, particularly with home-built apparatus. While interlocks, beam stops, and protective shutters are mandatory in commercial microscopes, custom set-ups are often more reminiscent of open optical bench systems and do not comply with legal guidelines. Hence, developers and experimenters should stay in close contact and new users should be briefed about risks.

3. Concluding Remarks

Neither does this introduction replace a careful reading of the original papers describing different imaging formats nor does it replace making your own experience with the equipment you bought. But it alerts the reader to consider some parameters that are not so obvious when looking at microscope brochures and reading the often very condensed "materials and methods" sections. If there is a simple conclusion, then it is this: know your microscope. It pays for your research and your next budget.

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