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Fluorescent Probes for Confocal Microscopy

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1. Introduction

This chapter differs from others in this volume in that it does not describe protocols *per se*. Rather, it consists of checklists, cautions, tips, rules of thumb, and advice related to fluorescent probes in general and probes for confocal microscopy in particular.

An excellent overview of fluorescence and fluorescence techniques can be found in the Introduction to the Handbook of Fluorescent Chemical and Research ***(1)***. The company's Web pages include probe spectra, application notes, and Material Safety Data Sheets (MSDSs), as well as an up-to-date version of their catalog ***(2)***. There are several lists of probes used in confocal laser scanning microscopy ***(3–6)***, and new dyes are constantly being developed (e.g., ***7,8,20***).

When specific probes or classes of probes are used as examples in the following sections, the information provided is necessarily incomplete; for detailed handling protocols, please consult the literature. Finally, some of this discussion pertains to single-photon excitation (confocal) microscopy only; in particular, the multiphoton excitation spectra of fluorophores are similar but not identical to their single-photon spectra (*see* **Note 1**).

1.1. General Considerations

1. Obtain a copy of the enclosures that come with the probe from the supplier before you order it (*see* **Note 2**).
2. Consult the published literature carefully *before* purchasing the probe (*see* **Note 3**).
3. To locate probe literature, utilize the specific indices (e.g., the CAS NO in Medline, *see* **Note 4**) available in electronic searches of bibliographic databases (*see* **Note 5**).

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4. Overall, the idea is to make sure you can in fact use the probe for the purpose you intend before you buy it.

1.2. Definitions

1. Fluorophores, fluorochromes, fluorescent dyes, and fluors all refer to molecules that fluoresce.
2. A fluorescent probe often implies a fluorophore attached to another molecule, although it can also mean the fluorophore itself.
3. The estimated fluorescence intensity of a fluorophore is the product of its quantum yield (Q_f, no units, typically 0.05–1.0) and its molar extinction coefficient (ε, $M^{-1} \bullet cm^{-1}$, typically $10^4 - 2 \times 10^5$; *see* **Note 6**) of the probe (assuming subsaturation excitation). However, both quantities are measured under specific conditions that do not usually pertain in a given experiment (*see* **Note 7**).

2. Materials

2.1. Selecting a Probe

In general, a useful fluorescent probe is one that associates specifically with the structure(s) of interest, provides a strong signal, photobleaches slowly, and is nontoxic. Important additional considerations in the selection of a probe for confocal microscopy are the laser lines available, the excitation and emission spectra of the probe, and the confocal filter sets that can be used to collect the fluorescence.

1. Choose a fluorophore that can be excited by the available laser lines; the absorption maximum of the probe (*see* **Note 8**) should be at or near one of the laser wavelengths available (*see* **Note 9**).
2. It is often useful to check the probe's emission spectrum in a solvent system that approximates the one in which it will be used (*see* **Note 10**).
3. Choose a probe with high fluorescence intensity (*see* **Note 11**); use of a low photometric gain will minimize noise (*see* **Note 12**).
4. When using more than one probe in the same preparation, try to choose fluorochromes with well-separated excitation and emission spectra and that have minimal emission overlap (*see* **Note 13**).
5. Verify that the fluorescence filters available can be used to collect the emission spectra of the probes with reasonable efficiency (*see* **Note 14**).
6. If the tissue will be fixed after exposure to the probe, choose a probe that can be fixed as well. Otherwise, it may be washed out during the fixation process (*see* **Note 15**).

2.2. Storing the Probe Upon Arrival

Fluorophores can be air, water, temperature, and/or light sensitive. Some are shipped as mixtures that will degrade over time (e.g., by hydrolysis).

1. Maintain a database of your probes (*see* **Note 16**).
2. Determine the necessary storage conditions before ordering the probe; finding room in a freezer to accommodate a desiccator is not always easy (*see* **Note 17**).
3. Do not automatically store all fluoroprobes in a freezer! Some probes (and certain probe components) should never be frozen (*see* **Note 18**).
4. If a dry fluor is hydroscopic, then minimize its exposure to the water vapor in air by dividing it into aliquots and storing these appropriately (e.g., under dry nitrogen).
5. It is often wise to check the purity of a probe upon arrival, especially when ordering from a new supplier (*see* **Note 19**).

2.3. Getting the Probe into Solution

As a rule, probes in aqueous solution are unstable (e.g., subject to slow hydrolysis), as well as being more light and heat sensitive than the dry form. It is usually best to store hydrophilic probes dry, and to put them into solution just before using them. Always protect a probe in solution from light!

1. Make a habit of using double gloves when handling probes (*see* **Note 20**).
2. Always use freshly prepared buffer; old or used buffer often gives poor staining.
3. For certain compounds (e.g., the salt form of some peptides and proteins, or of nucleotide derivatives), an exact molecular weight is not known, and the concentration of the probe solution should be determined post-preparation (e.g., by measuring the absorbance and using the extinction coefficient to calculate concentration) (*see* **Note 21**).
4. To get a probe into aqueous solution, try changing the pH slightly, adding a small amount of a mild detergent, and/or using strong mechanical agitation (*see* **Note 22**).
5. Remove dissolved oxygen from a solution that has been shaken or agitated by exposing the solution to vacuum for 1–2 min (*see* **Note 23**).
6. Check the probe solution for the presence of insoluble particles (*see* **Note 24**).
7. Calibration solutions for ion indicators must be free of heavy metal ions (*see* **Note 25**).
8. The buffer used for loading acetoxymethyl (AM) probes should be serum free, and should also not contain any primary or secondary amines (*see* **Note 26**).

2.4. Storing Probe Solutions

1. Lipophilic probes can often be safely stored frozen for a long time as a concentrated stock solution (*see* **Note 27**).
2. Allow a stock solution to thaw completely, rather than using the first portion to liquefy and then refreezing the remainder (*see* **Note 28**).
3. Proteinaceous probes (e.g., antibody conjugates) can aggregate during storage, and these aggregates can result in nonspecific staining. Similarly, some probes (e.g., propidium iodide) can precipitate when frozen (*see* **Note 29**).
4. The AM esters of many probes (e.g., fluo-3 and rhod-2) are very susceptible to hydrolysis (*see* **Note 30**) and should be assayed for decomposition before use if they have been stored improperly, or for longer than 6 months (*see* **Note 31**).

5. Check the purity of the probe before use, e.g., by TLC with an appropriate solvent system, particularly if the application requires that no free dye be present (*see* **Note 32**).
6. If you store probe solutions in scintillation vials, be aware that some dyes can react with the metal foil inside the cap (plastic-lined caps are available). Also note that glass binds some dyes more than plastic does, and thus glass storage containers are not always best for probe solutions (*see* **Note 33**).

3. Methods

3.1. Do a Background Check

Autofluorescence can be a significant source of artifact in confocal microscopy, and reduces the signal-to-noise ratio in an image. Biological autofluorescence is usually elicited by excitation wavelengths less than 500 nm; however some specimens (e.g., those that contain significant amounts of chlorophyll or porphyrins) will be autofluorescent in the far red. The nature and extent of autofluorescence can also depend on how the sample was prepared ***(9)***.

1. Avoid aldehyde fixation, particularly if glutaraldehyde is used, as this will induce autofluorescence. The same is true for picric acid and periodate (*see* **Note 34**).
2. It is often possible to reduce or eliminate autofluorescence by careful choice of filter cutoffs, or by use of high-wavelength probes (*see* **Note 35**).
3. Reduce or remove the signal from free or nonspecifically bound probe ("reagent background") as much as possible before imaging (*see* **Note 36**).
4. When using ratiometric probes, background fluorescence corrections must be made in order to calculate the ratio at any point.
5. Always check a test sample for autofluorescence after all preparative steps, with the setup that will be used for imaging (*see* **Note 37**).

3.2. Labeling

This section provides a few illustrations of precautions to be taken with fluors of interest in confocal microscopy. It is not intended to be inclusive.

1. The best temperature, exposure time, and probe concentration for probe loading must be determined by trial and error; however, in general, it is best to use as little of the dye as possible, and to load at room temperature (rather than at the optimal physiological temperature for the sample) (*see* **Note 38**).
2. Ion probes (e.g., pH, calcium) must be calibrated in a medium that closely mimics the experimental medium. An in situ calibration (permeablizing the cells) is usually the best way of accomplishing this (*see* **Note 39**).
3. When using AM esters of ion indicators (such as BCECF, fluo-3, and rhod-2), be sure to check for probe compartmentalization (*see* **Note 40**).
4. Increasing the concentration of an ion-indicator dye can buffer the ion of interest, and interfere with cell function (*see* **Note 41**).

5. Be very careful to ensure the complete mixture/dissolution of the probe in the working solution before it is used (*see* **Note 42**).
6. Conventional cell and tissue stains can interfere with the staining of the fluoroprobe and/or quench fluorescence.
7. After dye loading, be sure to wash the sample with buffer to remove any dye that is nonspecifically associated with cell membranes.
8. Finally, keep in mind that the probe may participate in the specimen's biochemistry in unexpected ways (*see* **Note 43**).

3.3. Detecting the Fluorescence Signal

1. Always use the minimum laser intensity possible to obtain a useable image.
2. Always collect an image using the full dynamic range available, but take care not to exceed it (saturation) or to fall below the detection threshold (*see* **Note 44**).
3. Increasing the amount of label present (i.e., adding more of the probe, but below the quenching concentration) can increase the detected intensity, but be aware that the effect is not linear.
4. Do not overexcite the probe with too high an illumination level (*see* **Note 45**).
5. When using multiple probes, check for bleedthrough from the lower channel into the upper. If bleedthrough is unavoidable, determine where it occurs (that is, which features labeled by the lower probe are detected in the higher channel) (*see* **Note 46**).
6. Be aware that when you are using dual (or multiple) probes, and the emission spectrum of the lower wavelength probe overlaps the excitation spectrum of the higher wavelength one, then you can have FRET (fluorescence resonant energy transfer) ***(10)*** (*see* **Note 47**).
7. Be alert to the possibility of label leaving the probe, or of the existence of artifactual fluorescent fragments (*see* **Note 48**).
8. Do not confuse detection with resolution (*see* **Note 49**).

3.4. Photobleaching

The total fluorescence signal (the brightness of the image) can become larger if the intensity of excitation and/or the collection time per pixel are increased, but in practice, photochemistry (including photobleaching) sets a limit to the amount of light that the specimen can be exposed to. Photobleaching is the irreversible destruction of a fluor by light over time, and is associated with the presence of molecular oxygen.

1. Check the rate of photobleaching on a noncritical portion of the sample before beginning imaging (*see* **Note 50**).
2. Use an antioxidant (antifade) agent in fixed preparations (*see* **Note 51**).
3. To minimize photobleaching in living preparations, reduce the partial pressure or concentration of oxygen (if the preparation can tolerate it) (*see* **Note 52**).
4. Software magnification ("zoom") in some confocals will increase the rate of photobleaching (*see* **Note 53**).

5. To obtain a Z-series in a thick specimen in which probe distribution is believed to be more or less uniform, but intensity diminishes rapidly with depth, consider collecting the images "backwards"; that is, taking the first section at the maximum depth, and then moving up toward the surface (*see* **Note 54**).
6. Phototoxicity considerations can be the determinant of which probe is best to use (*see* **Note 55**).

3.5. Environmental Effects

Fluorescence is strongly affected by the probe's environment, including solvent polarity (*see* **Note 56**), pH, and the presence of substances that quench fluorescence (including the probe itself). Quenching is a molecular interaction that reduces quantum yield.

1. The emission spectra of many probes depend on solvent polarity; in some cases, the probe is virtually nonfluorescent in water (*see* **Note 57**).
2. The excitation spectrum (as well as the emission spectrum) of certain probes (e.g., Nile red) depends on the polarity of the solvent.
3. For maximum fluorescence, fixed specimens stained with a pH-sensitive probe (e.g., fluorescein and some fluorescein derivatives) should be mounted in aqueous media with an appropriate pH (8 or greater for fluorescein) (*see* **Note 58**).
4. Do not assume that the pH of either a buffered probe solution or culture medium is stable over time — test it (*see* **Note 59**).
5. The pH of the probe solution and medium can affect staining efficiency and the stability of the probe as well as its fluorescence emission.
6. Proteins with aromatic amino acid residues can quench some fluors, notably fluorescein and 7-nitrobenz-2-oxa-1, 3-diazole (NBD) (*see* **Note 60**).
7. Self-quenching (or concentration quenching) occurs when the concentration of fluor is too high, and can also take place when multiple fluorophores label the same moiety (*see* **Note 61**).

3.6. Instrumental Concerns

Signal-to-noise ratio, resolution, and the overall image quality can also be affected by instrumental factors, some of which are discussed below.

1. Use a mounting medium with an index of refraction close to that of your sample; otherwise image quality will deteriorate rapidly as a result of the index mismatch (*see* **Note 62**).
2. Use an objective lens with a lower magnification and a high numerical aperture (NA), and employ software magnification ("zoom" on Bio-Rad confocals) rather than an objective lens with higher magnification and similar NA (*see* **Note 63**).
3. Verify that the microscope optics and detector to be used are appropriate for the probe (*see* **Note 64**).
4. The confocal filters should match the probe(s) used (*see* **Note 65**).
5. Check the optical alignment using your sample, and with all imaging elements (objective lenses, filters, etc.) to be used in place (*see* **Note 66**).

6. If you have an unexpectedly dim (or no) image, follow the lightpath from the sample in a stepwise fashion back as far as you can go, and look for mechanical sources of trouble (*see* **Note 67**).
7. If you check your sample using conventional excitation (e.g., looking for features of interest), don't be surprised if a high red label looks very dim (*see* **Note 68**).
8. Be careful to avoid the inadvertent inclusion of fluorescent material during the preparation or observation (e.g., as part of a sample holder) of the specimen (*see* **Note 69**).

3.7. An Example: FITC as a Confocal Probe

Fluorescein isothiocyanate (FITC) is arguably the most widely used fluorescent probe for the preparation of conjugates. Evaluating it in terms of the criteria listed previously:

It has the advantages of a high quantum yield, good absorption, and thus good fluorescence; an emission spectrum that does not shift significantly upon conjugation; excellent water solubility; low nonspecific binding; its maximum excitation (490 nm) is near the 488 nm line of the argon laser; its chemistry is well described, and many protocols for its use appear in the literature; and it is well detected using standard confocal filter sets.

It does, however, have significant disadvantages: its fluorescence is sensitive to pH and solvent polarity; its emission spectrum overlaps the biological autofluorescence range; the dye photobleaches readily, and is subject to quenching upon conjugation; and it has very long emission tail, which often bleeds through to the higher wavelength channel in multiprobe experiments.

FITC is, of course, a useful probe in confocal microscopy, but it is important for the investigator to be aware of its limitations.

4. Notes

1. Multi-photon excitation laser-scanning fluorescence microscopy is a promising new technique in which the specimen is illuminated by short, intense pulses of infrared (IR) light. At the point of focus, the photon density is sufficiently high so that two or more of these photons can be adsorbed essentially simultaneously by a fluorophore. When this occurs, it is as if a single photon having approximately the summed energy of the IR photons had been absorbed, and the fluorophore then returns to its ground state by the emission of a photon. In contrast to confocal (single-photon) imaging, excitation is restricted to the focal volume, and thus the volume in which photobleaching and other photochemistry take place (including the release of caged compounds) is greatly reduced. In addition, because all emitted light must originate from the focal volume, no aperture is required to eliminate out-of-focus light and the amount of light that can in principle be collected is greatly increased. For a short description of the technique, see the IMR web page ***(11)***.

   The multiphoton excitation spectra of fluorophores are similar but not identical to their single-photon spectra ***(12)***. Note also that UV fluorophores (e.g., indo-1)

can be excited by this method without using either UV illumination or optics corrected for UV. The method does have certain limitations: For a given fluorochrome, the spatial resolution using multiphoton imaging is slightly lower than that obtained using confocal imaging. Furthermore, if there exists a UV chromophore in the sample that absorbs at the excitation wavelengths, then there is a possibility of thermal damage to the specimen. However, the major obstacle to the wider use of the technique at present is the expense of the instrumentation, particularly the mode-locked laser.

2. The probe enclosure often provides more detail about use and handling than the description in the catalog does.
3. Don't depend exclusively on the references cited by the supplier. These are a good start, but are not necessarily the most relevant references for your application, and are often not up to date.
4. The CAS NO refers to the Chemical Abstracts number, a "unique" five- to nine-digit number that identifies a chemical substance. The number is usually assigned to the native form of a compound, but occasionally a salt form will receive a separate CAS NO. CAS numbers are also given to classes of substances, e.g. dextrans. In the case of enzymes, a number beginning with EC is assigned by the Enzyme Commission of the International Union of Pure and Applied Chemistry. In older entries, the RN (registry number) field is the CAS number.
5. An index is an identifier that is part of the database record entry for the citation (e.g., AU for author). Useful indexes in Medline (in addition to the CAS NO) include CH (chemical name) and XCH (exact chemical name). EC numbers can be searched as if they were CAS numbers, e.g., "FIND CAS NO EC2.7.1.2" retrieves citations about glucokinase. Displaying a Medline citation in full Medline format will show the contents of all the indices, including CAS NO and EC. Other databases, such as Biosis, can be searched using the chemical name as a keyword, but do not have specific chemical name indices.
6. The phycobiliproteins, with multiple fluorophores on each protein unit, typically have extinction coefficients on the order of $10^6\ M^{-1} \bullet cm^{-1}$.
7. Q_f, which may be taken as the ratio of photons emitted to photons absorbed by a fluorophore, integrates total photoemission over the fluorescence spectrum of the probe, whereas ε (the absorptivity of a 1*M* solution of the fluorophore through a 1-cm lightpath) refers to absorption at a single wavelength, usually the absorption maximum, in which case it may be identified as ε_{max}.
8. In nearly all instances, the fluorescence excitation spectrum for a probe is identical to its absorbance spectrum.
9. The excitation wavelength does not need to be exactly at the absorption maximum of the dye, as long as the extinction coefficient for the fluor at that wavelength is sufficiently large.
10. Published spectra and excitation/emission maxima are often measured using unphysiological solvents, and the emission spectrum of many probes will change as a function of the probe's environment. The excitation spectrum is less likely to do so, but there are exceptions; e.g., the excitation AND emission

spectra of Nile red are shifted to shorter wavelengths with decreasing solvent polarity.

11. The importance of using fluorescence intensity as opposed to quantum efficiency alone as a measure can be seen by noting that fluorescein ($\varepsilon \approx 70{,}000$, $Q_f \approx 0.9$) and Cy5 ($\varepsilon \approx 200{,}000$, $Q_f \approx 0.3$) have very different quantum efficiencies, but almost the same fluorescence intensity.
12. Although a probe's actual Q_f (and to some extent, its ε as well) are dependent on experimental conditions, probes tabulated as having high Q_f and high ε under analytical conditions are generally preferable to those with low numbers.
13. "Bleedthrough" — detection of some fluorescence from the shorter-wavelength probe in the longer wavelength channel — can to some extent be compensated, e.g., by weighted subtraction of one image from the other, but this is rarely completely successful ***(3)***.
14. General purpose ("one size fits all") filtersets may not be a good match to the spectra of your probes. Note also that if optical elements (including filters) must be moved or exchanged to image different fluorophores, this may create alignment problems — that is, the images may not be in perfect register.
15. For example, standard dextrans are not readily fixable, and may be washed out during the fixation process, but lysine-fixable versions of some dextran flow tracers can be obtained.
16. Include where the probe is stored in your laboratory, when it was received, the chemical name under which the MSDS is filed, the supplier, and the catalog number, as well as the molecular weight, storage conditions, etc.
17. To store probes, first wrap silver foil around the container, but not the around the cap. Make a separate "hat" for the cap that extends at least 1/2-inch past the top of the foil enclosing the container. This lets you open the container without the need to unwrap the foil each time. Repeated unwrappings and rewrappings will eventually result in the foil seal becoming damaged and no longer light-tight. Wrap a ring of white freezer tape (packaging tape intended for use in a freezer) around the foil enclosing the container, and use a permanent marker to record the name of the probe, the date of receipt, etc.
18. Examples include fluorescent low-density lipoprotein (LDL) complexes and streptavidin–alkaline phosphatase conjugate.
19. Probe purity varies widely between manufacturers, and sometimes between lots from the same supplier. TLC with an appropriate solvent system is a convenient way to access the purity of many probes. Note, however, that some molecules that are typically listed with a single molecular weight are in fact polydisperse, and normally have a range of molecular weights rather than a single molecular weight. Labeled dextrans are a good example, with '3000 MW' dextran having polymers with molecular weightss predominantly (but not exclusively) in the range 1500–3000.
20. Many are known (e.g., 4', 6-diamidino-2-phenylindole [DAPI], propidium iodide) or potential (e.g., those that bind with nucleic acids) mutagens, and should be handled with care. In addition, solvents such as dimethyl sulfoxide (DMSO) enhance the penetration of molecules into skin and other tissues.

21. That is, by using Beer's Law, $c = A/\varepsilon b$, where $A \equiv$ absorbance (unitless), $\varepsilon \equiv$ absorptivity (extinction coefficient, in $M^{-1} \bullet cm^{-1}$), $b \equiv$ path length (1 cm), and $c \equiv$ concentration of solution (M).
22. In more detail, try changing the pH slightly (e.g., using a mild alkaline buffer, such as 0.1 M bicarbonate); adding a small amount of detergent (e.g., Pluronic™ F-127, a non-ionic detergent; or Triton X-100); however, note that this may reduce staining efficiency, and that long-term storage in a detergent solution is not advisable; or mechanically agitating the solution strongly, e.g., by flushing the pipet several times, vortex-mixing, sonicating, or mildly heating (40–50°C) the solution.
23. Be sure to keep the solution protected from light, and make sure that the solution is exposed to the vacuum (e.g., the cap of the vial is loose). This is not advisable with a volatile solvent.
24. If any are present, remove them by centrifugation (e.g., in a microfuge at 12,000g for 5 min), by filtration through Whatman No. 2 filter paper, or by forcing the solution through 0.22-μm membrane filters.
25. Heavy metal ions (such as manganese) will affect both the indicator's affinity for the ion and its fluorescence. Metal chelators (e.g., tetraks-(2-pyridylmethyl) ethelenediamine [TPEN] for calcium) can sometimes be used to remove unwanted metal ions. Note also that significant amounts of metal impurities have recently been found in buffer components; make certain that the source certifies the salts as being high purity or effectively metal-free.
26. Serum often contains esterases, and aliphatic amines can cleave the AM esters and thus prevent loading.
27. The stock solution is typically 1–5 mM in an organic solvent; a working solution can then be made up just prior to use by diluting the stock (typically to 1–5 μM) in an appropriate buffer or solution. This saves time and avoids repeated exposure to moisture, air, etc. and the need to repeatedly freeze and thaw the parent probe solution to obtain a working solution. Smaller aliquots will also thaw faster.
28. The liquid that thaws first will often have a higher concentration of dye than the portion that is still frozen. When thawing is complete, the solution should be remixed and inspected for precipitate or evidence of aggregation (such as cloudiness). Always allow a cold solution to warm to room temperature before opening the container.
29. Remove aggregates by centrifugation, using only the supernatant for labeling, and check for the presence of precipitate after the solution reaches room temperature, either sonicating or vortex-mixing the solution to redissolve the dye.
30. Some probes, including the AM esters of fluo-3 and rhod-2, are sold in very small aliquots to avoid this problem.
31. To determine whether an AM ester is intact, dilute an aliquot of the DMSO stock solution to 2–10 μM in a cuvette containing 2.5 μM calcium test solution in buffer, measure the fluorescence, and then increase the calcium concentration to 5 μM and measure the fluorescence again. Because only the hydrolyzed form of the fluor will bind calcium, any increase in fluorescence indicates that partial

hydrolysis of the ester has taken place. The acid (hydrolyzed) form of the probe will not diffuse across the cell membrane.

32. Probes can also break down during storage; for example, labeled dextrans release small amounts of free dye over time, and must be repurified before use if the presence of free dye is not acceptable. Isothiocyanates such as FITC can also deteriorate during storage.
33. One example is SYBR Green, for which Molecular Probes recommends the use of Rubbermaid Servin' Saver® sandwich boxes.
34. If aldehyde fixation is necessary, the induced autofluorescence can be reduced by washing the specimen with 0.1% sodium borohydride in pH 8.0 phosphate-buffered saline (PBS) for 30 min before staining ***(9)***.
35. Narrowing the bandpass also reduces the fluorescence intensity detected; thus the use of high-red fluors is preferable. The use of long wavelengths does, however, raise questions about resolution and optical response ***(13)***.
36. For example, reagent background due to the leakage of fluo-3 (and other fluorescein derivatives) can be quenched by adding anti-fluorescein antibody to the external medium. The cells or tissue should also be washed with an appropriate buffer (e.g., the solution that the probe was made in) before imaging to remove any probe that is loosely or nonspecifically associated with the sample.
37. As noted in the text, fixation can induce autofluorescence, as can heating, addition of certain buffers, prolonged storage, and many other factors..
38. Some examples: dextran conjugates of ion indicators are less subject to compartmentalization and leakage, but must be loaded into the cell by invasive procedures, and often have a lower affinity for the ion being measured. The appropriate concentration of antibody conjugates needs to be determined by trial and error, but is usually between 5 and 20 μg/mL in most cases ***(14)***.
39. For SNARF-1, a ratiometric pH probe, an in situ calibration is performed by using an ionophore or other permeabilizing agent to equilibrate the intracellular pH with that of the known extracellular medium. Agents used to permeabilize cells include A-23187, ionomycin, nigericin, digitonin, and saponin.
40. Ion indicators are usually loaded into cells in the form of AM esters, which are subsequently cleaved by cytoplasmic esterases to form the fluorescent indicator, in this case BCECF*. If the AM form translocates to an intracellular compartment, it may still convert to the fluorescent form, but the sequestered indicator will not be responsive to changes in cytoplasmic ion levels.
41. A "dose–response" curve for probe loading in the particular system can be constructed to determine the maximum allowable loading concentration. Typical intracellular concentrations of probe range between 30 and 100 μ*M*.
42. Remove dissolved oxygen from a nonvolatile solution by subjecting it to a vacuum for 1–2 min, and check for the presence of insoluble particles.
43. For example, rhodamine 123 is a P-gp substrate. The point here is that the probe may not act solely as a probe in the system of interest.

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*2',7'-*bis*-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein

44. Although contrast adjustment is possible after the image has been obtained (and can make some features easier to see), the amount of information in the image remains the same. Similarly, all signal strengths out of range will be recorded as having the top level (e.g., 255), no matter what their actual intensity is.
45. The laser intensities in spot-scanning confocal microscopy can easily cause excitation saturation of a probe, especially those with long fluorescent decay times, such as the pyrenes. The result is that the detected intensity is no longer primarily a function of fluor concentration ***(15)***, and the signal-to-background ratio is reduced. A higher illumination level also means a higher rate of photobleaching.
46. Do so by taking (on the same area, if possible) a first image using the lower wavelength, a second image using the upper wavelength, and a third using both lines simultaneously. Merge images 1 and 2 and compare the result with image 3 (it may be necessary to manipulate intensities slightly in the merged image).
47. If the probes are sufficiently close together (10–100 Å, depending on the probe spectral characteristics), then the excited state of the lower wavelength probe can couple to the ground state of a proximate higher wavelength probe and excite it. In other words, the lower wavelength probe is excited, but it is the upper wavelength probe that emits.
48. High-intensity illumination (as well as cellular esterases) can cause some probes to break down, and the breakdown products themselves can be fluorescent. For example, the mitochondrial stain hexyl ester rhodamine B, which is excited by green wavelengths and has maximal emission at 578 nm, can be broken down by excitation light, and the breakdown products are fluorescent in the fluorescein range.
49. Resolution is limited by the imaging wavelength used — that is, a 200 nm liposome cannot be resolved using visible light. It can, however, be detected (if the intensity of fluorescence emission is sufficient). In analogy, the naked eye can detect stars at night, but cannot resolve a stellar disk — they appear as point sources. Note that for the liposome, this also means that you cannot distinguish between an intact liposome and a labeled fragment.
50. Adjustment of laser intensity and gain is far easier before the critical images are taken.
51. Although it is by no means the only good photoprotectant ***(16,17)***, 0.1% *p*-phenylenediamine (Sigma Chemical Co.) in 10% PBS and 90% glycerol (or in a buffered solution) is an excellent antifading agent for confocal microscopy ***(18)***. It is, however, carcinogenic and should be handled with extreme care. For living specimens, antioxidants such as ascorbic acid (0.1–1.0 mg/mL, or greater) have been used in the medium as reducing agents. Keep in mind that the primary label and the counterstain (if any) can affect the performance of an antifade reagent.
52. For example, by bubbling nitrogen or argon over it. The use of an enzyme that depletes oxygen in the medium (Oxyrase, Oxyrase Inc., P.O. Box 1345, Mansfield, OH 44901) has also been reported.
53. If the scan of the smaller ("zoomed") image takes the same amount of time as scanning a full-frame, then the total energy delivered per unit area in the small

image is larger than that for a full frame, and there is consequently more photobleaching. One method of avoiding this effect is to first collect a full-frame image and store it, and to then to magnify the digitized image.

54. In a point-scanning confocal, photobleaching occurs throughout the specimen, not just in the optical section being imaged. If the series begins at the top of the specimen, then the deepest section may be photobleached before it can be imaged. By starting deep, and moving progressively back toward the surface, it is the more superficial sections, with their higher intensity, that undergo the most photobleaching.
55. For example, the long-wavelength calcium indicator Calcium Green-1 has been shown to be less phototoxic than fluo-3.
56. "Solvent" here is interpreted to include the interior of any membrane or membrane-bound structure, as well as regions inside molecules.
57. Nile red, ethidium homodimer, the carbocyanines, NBD, propidium iodide, ethidium bromide, and diphenylhexatriene (DPH) are all strongly polarity sensitive. They have a low quantum yield in aqueous solution, and become strongly fluorescent when they are shielded from water by entering a hydrophobic environment or binding to a biomolecule.
58. Fluorescein has a pK_a of about 6.4, and its fluorescence emission drops rapidly as the medium becomes more acidic. Nearly all of its derivatives display some pH sensitivity as well.
59. For example, the pH of Tris buffers increases when they are refrigerated; if an 8.0 buffer is prepared at room temperature, the pH will increase to 8.5 at 4°C. Culture medium pH is affected by both temperature and CO_2 level.
60. This effect can be partially overcome in some cases; e.g., incubating fluorescein–avidin conjugates with free biotin at the end of the staining results in significantly brighter signals from the conjugates ***(19)***.
61. For fluorescein, the maximum brightness is obtained with two to four fluorophores per antibody.
62. Use a water lens to image aqueous (living) specimens, and a mounting medium with an index near that of the immersion oil used for fixed specimens. Matching optical indices is critical for high-resolution applications.
63. The intensity of the light collected increases as the square of the NA, but decreases as the square of the magnification. Specifically, a 60×/1.4 lens is preferable to a 100×/1.4 lens. Higher magnification lenses also tend to be "darker," due primarily to the large number of optical elements within them that the light must pass through.
64. UV probes require UV objectives and UV-reflective mirrors ***(4)***. Many older systems use photomultiplier tubes (PMTs) with very poor high red sensitivity, and optics that block or do not adequately correct high red wavelengths ***(13)***.
65. Ideally, the bandwidth of the emission filter should span 80–90% of the emission spectrum of the probe.
66. For dual or triple imaging, microspheres that are fluorescent at the wavelengths of interest are a convenient means of checking the registration of the channels

relative to each other. In brief, the microsphere images from the different channels are merged to see if they exactly superimpose. This procedure is described in more detail in the Handbook of Fluorescent Probes and Research Chemicals ***(2)***.

67. In our setups, these have included: internal diaphragm in objective stopped all the way down, partially or complete closed shutters and/or apertures, and presence of light-blocking filters in the lightpath.
68. The upper limit of human vision is about 750–770 nm, and retinal sensitivity above 650 nm is poor. A substantial part of the fluorescence emitted by some far-red fluors (e.g., the cyanines, such as Cy-5) is thus effectively invisible to the unaided eye. In other words, it is not possible to accurately judge the intensity or, in some cases, the presence of a high-red label by looking through the eyepieces of a conventional fluorescence microscope. However, most confocals can "see" farther into the red range than can the human eye.
69. Saran Wrap™, Canada balsam, various brands of clear fingernail polish, some epoxy resins, and certain machine oils (used to prevent the corrosion of sharp tools, such as razor blades) are fluorescent.

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References

1. Johnson, I. (1996) Introduction to fluorescence techniques, in *Handbook of Fluorescent Probes and Research Chemicals*, 6th edition (R. Haugland, ed.), Molecular Probes, Inc., Eugene, OR, pp. 1–6.
2. URL for Molecular Probes: http://www.probes.com/lit/
3. Brelje, T. C., Wessendorf, M. W. and Sorenson, R. L. (1993) Multicolor laser scanning confocal immunofluorescence microscopy: practical application and limitations, in *Cell Biological Applications of Confocal Microscopy* (B. Matsumoto, ed.), *Methods in Cell Biology*, Vol. **38**, Academic Press, San Diego, pp. 98–182..
4. Wells, S. and Johnson, I. (1994) Fluorescent labels for confocal microscopy, in *Three-Dimensional Confocal Microscopy: Volume Investigation of Biological Specimens* (J. K. Stevens, L. R. Mills, and J. E. Trogadis, eds.), Academic Press, San Diego, pp. 101–131.
5. Tsien, R. and Waggoner, A. (1995) Fluorophores for Confocal Microscopy, in *The Handbook of Biological Confocal Microscopy* (J. Pawley, ed.), Plenum Press, New York, pp. 267–280.
6. A list of probes that can be excited by laser wavelengths: http://flosun.salk.edu/fcm/fluo.html
7. Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R., and Piston, D. W. (1997) Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* **73,** 2782–2790.

8. Millard, P. J., Roth, B. L., Thi, H. P., Yue, S. T. and Haugland, R. P. (1997) Development of the FUN-1 family of fluorescent probes for vacuole labeling and viability testing of yeasts. *Appl. Environ. Microbiol.* **63,** 2897–2905.
9. Bacallo, R., Kiai, K. and Jesaitis, L. (1995) Guiding principles of specimen preservation for confocal fluorescence microscopy, in *The Handbook of Biological Confocal Microscopy* (J. Pawley, ed.), Plenum Press, New York, pp. 311–326.
10. Chapple, M. R., Johnson, G. D. and Davidson, R. S. (1988) Fluorescence quenching of fluorescein by R-phycoerythrin. A pitfall in dual fluorescence analysis. *J. Immunol. Methods* **111,** 209–218.
11. A short description of multi-photon microscopy: http://www.bocklabs.wisc.edu/imr/facility/2p.htm
12. Xu, C., Zipfel, W., Shear, J. B., Williams, R. M., and Webb, W. W. (1996) Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy. *PNAS* **93,** 10,763–10,768.
13. Cullander, C. (1994) Imaging in the far-red with electronic light microscopy: requirements and limitations. *J. Microscop.* **176,** 281–286.
14. (1995) Short protocols in molecular biology: a compendium of methods, in *Current Protocols in Molecular Biology*, 3rd edition (F. M. Ausubel, ed.), John Wiley & Sons, New York.
15. Pawley, J. (1995) Fundamental limits in confocal microscopy, in *The Handbook of Biological Confocal Microscopy* (J. Pawley, J., ed.), Plenum Press, New York, pp. 19–38.
16. Biloh, H. and Sedat., J. W. (1982) Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl galate. *Science* **217,** 1252–1255.
17. Johnson, G. D., Davidson, R. S., McNamee, K. C., Russell, G., Goodwin, D., and Holborow, E. J. (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immunol. Methods* **55,** 231–242.
18. Schuman, H., Murray, J. M., and DiLullo, C. (1989) Confocal microscopy: an overview. *BioTechniques* **7,** 154–163.
19. Emans, N., Biwersi, J., and Verkman, A. S. (1995) Imaging of endosome fusion in BHK fibroblasts based on a novel fluorimetric avidin-biotin binding assay. *Biophys. J.* **69,** 716–728.
20. Griffin, B. A., Adams, S. R., and Tsieng, R. Y. (1998) Specific covalent labeling of recombinant protein molecules inside live cells. *Science* **281,** 269–272.