Artifacts of light

The dangers of phototoxicity in fluorescence microscopy experiments are too often ignored.

Fluorescence microscopy has become an indispensible technique in life sciences research. Light is relatively noninvasive and is capable of penetrating intact living samples and reading out information from fluorescent dyes and tags that provide molecular specificity. But fluorophores require high irradiation intensities to generate usable signals. These intensities—especially at the shorter wavelengths required by most probes—generate toxic free radicals from exogenous dyes and endogenous chromophores, damaging cellular components and eventually resulting in cell death. Fluorescence imaging is therefore hardly an ideal noninvasive technique.

Phototoxicity has long been recognized as a potential problem and is often cited as a severe limitation for super-resolution microscopy techniques that deliver light dosages far in excess of those used in conventional imaging. Conversely, light-sheet fluorescence microscopy has been promoted for its ability to dramatically lower the light dosage required for volumetric time-series imaging.

Because most cells are adapted to cope with some level of light-induced damage, they have mechanisms to mitigate some phototoxic effects. But what is the threshold above which cells can't cope? It will undoubtedly vary by specimen, but a study in yeast showed that irradiation intensity had to be 100× lower than that in a typical microscope to avoid growth arrest or a substantial reduction in the number of cell doublings 20 hours after imaging (P.M. Carlton *et al.*, *Proc. Natl. Acad. Sci. USA* **107**, 16016–16022, 2010). Studies on mammalian cells have shown similar effects.

Most researchers probably do examine cells for obvious signs of phototoxicity, such as formation of membrane blebs or cell death, but measurements of cell-division rates for an extended period at the end of the experiment are certainly the exception and often infeasible. Less extreme effects of phototoxicity—but those sufficient to affect experimental results—are likely to go unnoticed, often because researchers don't know to look for them.

Cells that are already stressed are more sensitive to phototoxicity, so standard 'no treatment' controls may be insufficient in perturbation experiments. An observed phenotype could result from a phototoxic effect that isn't present in the untreated (and nonstressed) control. If an experiment has an observable macro effect that doesn't require the probe signal, an unlabeled and unimaged sample may serve as an appropriate control. But when the only readout is a molecular response communicated by the fluorescent label, controls are less straightforward. In these cases, examination of results from at least two different irradiation levels may be the best method to reveal light-dependent effects. It has also been noted that cells vary in their individual responses to irradiation, so high variability in data from imaged cells could also be a sign of phototoxicity.

We analyzed the full HTML text of original research articles published in a selection of Nature journals between 1 January 2005 and 3 November 2013 to try to determine whether researchers, reviewers and editors were giving sufficient consideration to phototoxicity. Compared to the numbers of articles mentioning terms related to fluorescence microscopy—and, therefore, expected to contain such data—tiny numbers of articles mention phototoxicity (*Nature*, 0.4%; *Nature Cell Biology*, ~1%; *Nature Immunology*, 1–2%; *Nature Neuroscience*, 2–3%; *Nature Methods*, 5–6%), which suggests that phototoxic effects were not taken into account or at least not discussed, except possibly in the supplement. Although this was a crude investigation with many caveats, it is likely that light-induced effects are having an unappreciated impact on reported experimental results.

Work to improve the photostability of fluorescent proteins could, perversely, contribute to phototoxicity problems. These probes, unlike many small-molecule dyes, contribute little to phototoxicity, but their resistance to photobleaching can encourage the use of higher irradiation levels, which then interact with endogenous chromophores to generate photodamage.

Improved probe quantum efficiency is highly desirable for increasing the efficiency with which illumination light is converted into a fluorescent signal and thus increasing the signal-to-noise ratio. But these probe improvements may best be viewed as an opportunity to lower irradiation levels without sacrificing signal. Researchers should certainly resist exploiting the improved photostability that often accompanies higher quantum efficiency to increase irradiation levels. The development of improved far-red and infrared fluorescent proteins that use less-damaging, longer-wavelength illumination is of utmost importance.

Regardless of the imaging technique or probe used, researchers must be more cognizant of the potential for phototoxicity to alter experimental results. Even light-sheet microscopy can cause severe phototoxic effects in spite of delivering far less light than other techniques during time-lapse three-dimensional imaging, not least because users push application boundaries. Appropriate controls will become ever more important as applications of fluorescence microscopy continue to expand and quantitative imaging is used to investigate ever finer and more sensitive processes and pathways.