OPEN

Excitation Light Dose Engineering to Reduce Photo-bleaching and Photo-toxicity

Received: [illegible]
Accepted: 12 July 2016
Published: 03 August 2016

Colton Boudreau[1,*], Tse-Luen (Erika) Wee[1,2,*], Yan-Rung (Silvia) Duh[3], Melissa P. Couto[3], Kimya H. Ardakani[2] & Claire M. Brown[1,2]

[illegible]
Similarly, in living cells, rapid line scanning resulted in reduced cell stress as measured by mitochondrial
[illegible]
laser scanning microscope, without al [illegible] hardware upgrades. Researchers can use the rapid [illegible]

As a substitute for oxygen depletion it has been suggested that pulsing excitation light results in a dramatic reduction in photo-toxicity[17–19]. For example, delivering more frequent pulses of lower energy multi-photon laser light rather than one pulse of higher energy can improve light output and decrease photo-bleaching and photo-damage[20]. eory has also been developed to suggest that scanning a confocal laser at high speed (e.g. resonant laser scanning confocal) should increase uorescence yield and reduce photo-bleaching[21]. During the uorescence excitation process when uorophores enter the excited singlet state there is a nite, albeit low, probability that they will enter the "forbidden" triplet state. e triplet state, o en called the "dark-state", is long lived as it decays on the microsecond time scale versus the nanosecond timescale of the singlet state decay. If molecules in the triplet state absorb additional photons they have enough energy to break covalent bonds. ese chemical reactions involve oxygen and can result in the release of reactive oxygen species. Highly reactive oxygen radicals can then cause further photo-bleaching and photo-damage, and cause photo-toxicity in living samples. In studies

In order to demonstrate that this was not a phenomenon limited to EGFP similar experiments were conducted with CHO-K1 cells expressing mCherry paxillin. As with EGFP, mCherry showed a signi cant reduction in photo-bleaching (~50%) with rapid line scanning (Supplemental Fig. 2).

induced photo-toxicity. Visualization of mitochondrial morphology is a good metric because mitochondrial s-sion from a complex extended network into small vesicular mitochondrial fragments is an early indicator of cell stress[25]

Discussion

With the array of available tools, live cell uorescence imaging is now commonplace and spans research elds in the physical, life and health sciences. However, most cells are never exposed to light during their lifetime and it is well known that high levels of light can induce toxic e ects on living systems. erefore, it is important to understand how light is delivered to the living specimen and how that may a ect cell health. In this study, xed and live cell assays were used to show that engineering the method of light delivery to the specimen in time can have a drastic e ect on uorophore photo-bleaching and live cell photo-toxicity.

It was not surprising that maintaining the same length c1e4(e)0.5(n iM.5()1(a)8.9(a)8.9(in)18.9(te)3.9(if)8.9(et) t)-6(h)8(

image quality. With the advent of tissue clearing and rapid super-resolution techniques reduced photo-bleaching will be important for high resolution imaging of large samples and for 3D time-lapse imaging of living samples.

e pixel dwell time for 8-line and 16-line scan settings is shorter than the lifetime of the triplet state of EGFP. erefore, it is likely that the drastic reduction in photo-bleaching is related to a low probability of triple state molecules to absorb additional light energy with microsecond or sub-microsecond light pulsing. e time between line scans was on the millisecond timescale giving any triplet state molecules adequate time to relax back to the ground state before the next rapid line scan. e involvement of the triplet state in the photo-bleaching process is supported by the minimal photo-bleaching measured in the presence of oxygen scavengers (Supplemental Fig. 1). More work needs to be done to determine if each uorophore will have an ideal pixel dwell time for a given laser power to minimize photo-bleaching.

Many other methods of light delivery to the sample, aside from the standard CLSM, could possibly reduce photo-bleaching as well. Similar experiments comparing wide eld and spinning disk illumination showed that reduced photo-bleaching can result from the spinning disk "pulsing" with a di erent microscope architecture of light delivery to the sample (Supplemental Fig. 3). Additionally, theory shows that the resonant scanning CLSM should see similar reductions in photo-bleaching and photo-toxicity[21]. To our knowledge there has not been a systematic laboratory study of these systems. Given the results shown here further studies will most likely demonstrate that the resonant scanning CLSM will show similar reductions in uorophore photo-bleaching and cellular photo-toxicity.

e live cell assays presented here, speci cally the cell protrusion assay, demonstrates that moving to rapid line scanning also results in reduced photo-toxicity. us, for any researcher performing live cell CLSM experiments they should immediately move away from single slow line scanning to averaging of multiple rapid line

ese studies show how a careful and systematic study of how light is delivered to the sample can result in marked reductions in photo-damage without any compromise in image quality. However, these studies have just begun to explore the possibilities of engineering light delivery to the sample in space and time such that they minimize hazardous sample conditions and maximize experimental output. Modern microscopes come with a plethora of designs and choosing how the light is delivered to the specimen could be critical for reproducible and accurate live cell experiments. e protrusion assay is straightforward to implement and should prove useful as a metric for photo-toxicity of any uorescence microscopy technique. It would be interesting to apply the technique to a more in depth study of spinning disk confocal microscopy, resonant confocal laser scanning microscopy, light sheet microscopy[27] and the super-resolution techniques. As live cell applications become more and more prevalent, the tools presented here will be essential to ensure procedures and protocols are optimized so the processes under investigation are not unduly in uenced by uorescence imaging.

Methods

Sample Preparation. Fibronectin (Sigma, F-0895) coating of 35 mm coverglass bottom dishes (World

solution for up to two weeks. Chinese hamster ovary K1 (CHO-K1) cells stably expressing paxillin-EGFP or

MitoTracker Red CMXRos (ermoFisher Scienti c, M-7512) for 30 minutes at 37 °C immediately before exper-

so ware (Northampton, MA). Where a_1 and a_2 are the relative amplitudes of each decay component and R_1 and R_2 are the decay rates for each component.

$$f(x) = offset + a_1 e^{-xR_1} + a_2 e^{-xR_2} \quad (1)$$

For easier comparison between experiments performed with di erent laser powers or di erent laser lines (e.g. 473 nm vs 488 nm), photo-bleaching rates were expressed in terms of the number of images collected and were

12. Carlton, P. M. *et al.* Fast live simultaneous multiwavelength four-dimensional optical microscopy.