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A CROSS BEAM EXCITATION GEOMETRY FOR LOCALIZATION MICROSCOPY

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Fluorescence microscopy is popular for its noninvasive properties and its use in imaging multiple species simultaneously (1). Furthermore, superresolution fluorescence localization microscopy methods such as fluorescence photoactivation localization microscopy (FPALM) utilize photoactivatable, photoswitchable, and photoconvertible fluorescent proteins to improve the lateral resolution of conventional fluorescence microscopy by an order of magnitude (2-4). However, our understanding of the effects of excitation light polarization coupled to the transition dipole orientation of fluorescent labels within the sample is incomplete. Specifically, the excitation rate of a given label is proportional to $\cos^2\theta$, where θ is the angle between the excitation illumination polarization and the transition dipole moment of the label(1). Because of this relationship between the laser polarization and transition dipole orientation, using widefield circularly-polarized illumination, one cannot excite all possible fluorophore orientations simultaneously with equal efficiency. Since thresholds are commonly used in localization microscopy during the stage of identification of fluorophores, the number of localized molecules can also be reduced by such polarization effects. Here, we briefly present an experimental excitation geometry called cross-beam, which uses two lasers with similar wavelength to excite labels with any transition dipole orientation.

Figure 1A demonstrates the difference between widefield and cross-beam excitation. One excitation wavelength is circularly-polarized, and the other is linearly polarized, such that any dipole orientation can in principle be excited. Despite the differences in wavelength, excitation rates for the two beams are matched using the published extinction coefficient for the label. Furthermore, if these excitation rates are matched, one can in principle excite all probe orientations with the same rate, significantly narrowing the histogram of molecular emission rates, and allowing emission rate to be interpreted more meaningfully. Thus, the cross-beam orientation has both the potential to increase the number of localizations in a sample and the ability to excite each fluorophore at a more uniform rate than a widefield illumination geometry.

Figures 1B and C show an increase in the number of localizations as well as excitation rate uniformity using cross-beam excitation. In principle, one could use this method to image fluorophores that have similar emission spectra but different emission rates. Maximization of the number of localizations improves the sampling of structures of interest within the

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sample, which has been shown to be crucial for obtaining the highest resolution images possible (5, 6); (F. Pennacchietti et al., *Biophysical Journal*, *in press*). These improvements suggest that use of a cross-beam excitation geometry will lead to higher optimal resolution and new applications of superresolution microscopy in the future.

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