Applying spectral fingerprinting to the analysis of FRET images

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Förster resonance energy transfer (FRET) allows one to study interactions between two fluorescently labeled molecules (donors and acceptors) at distances on the order of 5 nm. Many studies have described methods of how to measure the efficiency of FRET. However, few have addressed the question of how fluorescence from unpaired donors and acceptors can be determined in addition to that from FRET-pairs and how the signal-to-noise ratio (SNR) of such estimates depends on the presence of the partner species. Such knowledge, however, is essential for many biological applications, in which-after initial characterization of the spectral properties of a well-defined donor-acceptor complex-the in vivo affinity and stoichiometry of the complex is of interest. Here, we provide a theoretical analysis on how spectral fingerprinting can be applied to separate fluorescence of FRET pairs from that originating from unpaired donors and acceptors and how to select imaging parameters to optimize the SNR of the estimates. Thereby, we uncover a fundamental problem in this application and discuss ways to evade its adverse consequences. We compare the expected resolution of traditional FRET measures with that of optimized spectral fingerprinting and analyze the resolution of a method for FRET measurements that combines spectral with fluorescence lifetime information.
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