



## How to measure separations and angles between intramolecular uorescent markers

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How to measure separations and angles between intramolecular fluorescent markers

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We demonstrate a novel, yet simple tool for the study of structure and function of biomolecules by extending two-colour co-localization microscopy to fluorescent molecules with fixed orientations and in intra-molecular proximity. From each colour-separated microscope image in a time-lapse movie and using only simple means, we simultaneously determine both the relative (x,y)-separation of the fluorophores and their individual orientations in space with accuracy and precision. The positions and orientations of two domains of the same molecule are thus time-resolved. Using short double-stranded DNA molecules internally labelled with two fixed fluorophores, we demonstrate the accuracy and precision of our method using the known structure of double-stranded DNA as a benchmark, resolve 10-base-pair differences in fluorophore separations, and determine the unique 3D orientation of each DNA molecule, thereby establishing short, double-labelled DNA molecules as probes of 3D orientation of anything to which one can attach them firmly.