PHOTON STATISTICS IN FLUORESCENCE ANISOTROPY IMAGING

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Anisotropy imaging can be used to image resonance energy transfer between pairs of identical fluorophores and thus constitutes a powerful tool for monitoring protein homo-association. The requirement for only a single fluorophore simplifies preparation and interpretation. We describe quantitative methods for the acquisition and the image processing of anisotropy data that return a per pixel estimate of the uncertainty of anisotropy. The analysis methods include calibration procedures and allow for a balance in spatial, anisotropy and temporal resolution.

In fluorescence, steady-state photon emission and detection obey Poisson statistics, leading to a fundamental uncertainty in the measured anisotropy. The experimental r is given by

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}\,, \tag{1}$$

where G is a system dependent factor accounting for the different sensitivities in the detection channels for $I_{\parallel}$ and $I_{\perp}$. We apply standard propagation of error assuming uncorrelated parameters. Combining this with eq. 1 we arrive at

$$var(r) = \frac{(1-r)(1+2r)(1-r+G(1+2r))}{3I_{tot}}\,, \tag{2}$$

where $I_{tot} = I_{\parallel} + 2GI_{\perp}$ is the total emission intensity. This can be of significance especially for confocal images where typically only a few dozen photons are detected per pixel. The analysis of the expected variance in a measurement due to photon statistics assumes that the measured signal intensity is given in collected photon counts, whereas instruments report ADU. A relation can be made if the gain is known or measured from a calibration image.

Based on this, filtering can be applied to $I_{\perp}$ and $I_{\parallel}$ in order to average photons from different spatial and/or temporal locations to decrease the uncertainty in the anisotropy while loosing spatial/temporal resolution. The filtering has to be performed on the measurement images and not on the result of eq. 1 as the mean of a ratio is a biased estimator in ratio imaging. To detect small spatial or temporal changes in anisotropy it is sometimes desirable to compute an image with constant uncertainty or alternatively a constant coefficient of variation. This can be achieved by building a Gaussian scale space with its strong causality constraint from the two input images $I_{\parallel}$ and $I_{\perp}$. The target standard deviation or CV can be compared to the computed one at a certain scale and for the pixels that match the criterion the anisotropy value is stored along with the respective scale. The scale selection can be performed with some prior knowledge.