

HOMO-FRET IMAGING QUANTIFIES SUBCELLULAR PROTEIN CLUSTER SIZES

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Fluorescence anisotropy based homo-FRET imaging can be applied to quantify the degree of clustering of identical proteins in cells. For this purpose, often controlled photobleaching or fractional labeling experiments are used. These approaches provide an indirect measure of the cluster size; the results of multiple samples and/or cells have to be combined for one cluster size determination. Here, we present a methodology where the measured anisotropy is directly related to average number of fluorophores per cluster. It therefore allows direct quantification of the degree of clustering of protein on a subcellular level.

Various experimental approaches were validated; steady-state and time-resolved anisotropy detection methods employing both one and two photon excitation. For the validation special constructs of green fluorescent protein (GFP) were used that either dimerize or oligomerize by addition of a ligand.

It is shown that independent on the method, the commonly made assumption of complete depolarization after a single energy transfer step is not valid here. This is likely due to a restricted orientation distribution of the fluorescent proteins. The relation between anisotropy and cluster size was therefore established experimentally. Measurements of dimers and oligomers of FKBP12 fused GFP yielded calibration curves that were used in quantitative cluster size imaging experiments.

The methodology was applied to study clustering of signaling proteins in the plasma membrane. For the epidermal growth factor receptor (EGFR), time-resolved anisotropy imaging reveals that small clusters are present before activation. After activation, the cluster size increases dramatically: anisotropy decreases to a level that corresponds to oligomerization (see Figure).

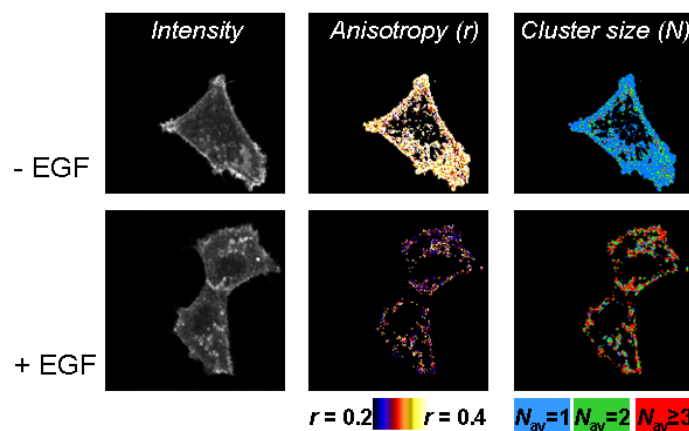


Figure: Intensity, (time resolved) anisotropy and cluster size image of H14 fibroblasts expressing EGFR-mGFP, before (“- EGF”) and after (“+ EGF”) activation.