Determining the geometry of epidermal growth factor receptor oligomers via fluorophore localization imaging with photobleaching.

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Introduction

Dimerisation, oligomerisation and clustering of cell surface receptors are recognized as important in the control of signalling processes. The epidermal growth factor receptor (EGFR, or HER1/ErbB1), one of a family of four receptors, is a cell-surface receptor tyrosine kinase that initiates signalling pathways responsible for cellular metabolism, growth and differentiation[1]. Dysregulation via overexpression or mutation to members of the human epidermal growth factor receptor (HER) family can lead to the development of various cancers[2]. The majority of EGFR research has largely focused on a dimerisation-dependent activation mechanism, however recent analyses suggest that oligomerisation also plays a crucial role in EGFR signalling but the structures and functions of the oligomers are poorly understood.

Although progress has been made in imaging distances between molecules in cells below the diffraction limit using FRET and super-resolution microscopy, methods for determining separations in the 10-50 nm range have been elusive. Members of the OCTOPUS[3] team have developed Fluorophore localisation imaging with photobleaching (FLImP)[4,5], based on the quantised bleaching of individual protein-bound dye molecules, to quantitate the molecular separations in oligomers and nanoscale clusters. Given the ~11 nm lateral dimension of active EGFR dimers the dimensions of higher order oligomers are expected to fall in the range appropriate for FLImP experiments. Here we used the FLImP method to probe the structure of inactive and ligand-induced EGFR oligomers measuring discrete pairwise separations between fluorophore conjugated HER1 Affibody or EGF. The work described here is part of work published recently in Needham *et al.* (2016)[6].

Materials and Methods

Chinese Hamster Ovary (CHO) cells expressing wild-type EGFR under an inducible Tet-ON promoter were a gift from Prof Linda Pike (Washington University). Cells were grown in 5% CO_2 in air at 37°C in phenol-red-free DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 1% penicillin-streptomycin, 100 mg/ml hygromycin and 100 mg/ml geneticin.

Briefly 1 x 10^5 cells were seeded on 1% BSA-coated Piranha cleaned 35mm no. 1.5 (high tolerance) glass-bottomed dishes (MatTek Corporation, USA) in 2 ml of media plus 50 ng/ml of doxycycline hyclate (Sigma), resulting in expression of ~10^5 receptors per cell. After 48 h the medium was changed to 0.1% serum plus 50 ng/ml doxycycline for 2 h. CHO cells were rinsed with PBS and cooled to 4°C for 10 min and then labelled with 4 nM EGF-CF640R or Affibody-CF640R for 1 h at 4°C. The N-terminus of EGF was labelled at a 1:1 ratio by Cambridge Research Biochemicals (Cleveland, UK). The EGFR Affibody was labelled at a 1:1 ratio at its single cysteine residue. Cells were rinsed and fixed with 3% paraformaldehyde plus 0.5% glutaraldehyde for 15 min at 4°C, then 15 min at room temperature. We used an Axiovert 200M microscope with TIRF illuminator (Zeiss, UK), with a x100 oil-immersion objective (α-Plan-Fluar, NA=1.45; Zeiss, UK) and an EMCCD (iXon X3; Andor, UK). The microscope is also equipped with a wrap-around incubator (Pecon XL S1). Samples were illuminated a fibre-coupled laser combiner (Andor) with a 100mW 640 nm diode laser (Cube, Coherent). Images were collected every 0.28 s. Empirical posterior FLImP distributions were then obtained based on discrete EGF or Affibody separation measurements that had confidence intervals of less than 6–7 nm. A more detailed description of the FLImP method can be found in refs 4-6 and is illustrated in Figure 1.

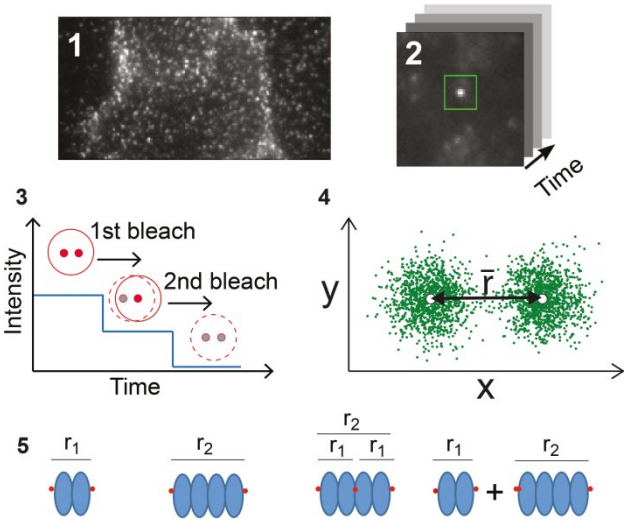

Figure 1. How to obtain separations with the FLImP method. (1) TIRF images are collected of fluorophore tagged ligand bound receptors and (2) spots of individual complexes are tracked to give intensity time courses. (3) Representation of a spot containing two fluorophore-conjugated ligands (red dots) where the intensity v time trace shows two intensity levels which decays to zero in two bleaching steps. When one fluorophore bleaches (grey spot) the centroid position of the spot shifts. (4) A global least-squares seven-parameter-fit is used to identify the best intensity, x-y positions and the full-width at half-maximum of the point spread function for each fluorophore, from which their separation is calculated with a precision determined by the localization error; (5) Examples of the separations collected from a two-ligand dimer and tetramer, a three-ligand tetramer, and a mixture of a dimer and a tetramer.

The structure of active EGFR complexes.

FLImP measurement of the separation of a pair of fluorophore-conjugated EGF ligands bound to an EGFR complex produces an empirical posterior probability distribution of the separation,

taking the form of an asymmetric Rice distribution. The posterior distribution width (or 69% confidence interval) reflects the precision of each pairwise separation measurement. The posteriors with 69% confidence intervals smaller than the required resolution (typically 4–7 nm) are retained and pooled into a histogram known as a FLImP distribution (Figure 2). These can be used to derive structural information of EGFR complexes bound to more than one fluorophore tagged ligand. An estimate of the proportion of measurements consistent with species of EGFR complexes bound to more than one EGF ligand as an indicator of the relative population of the species can also be deemed (Figure 1).

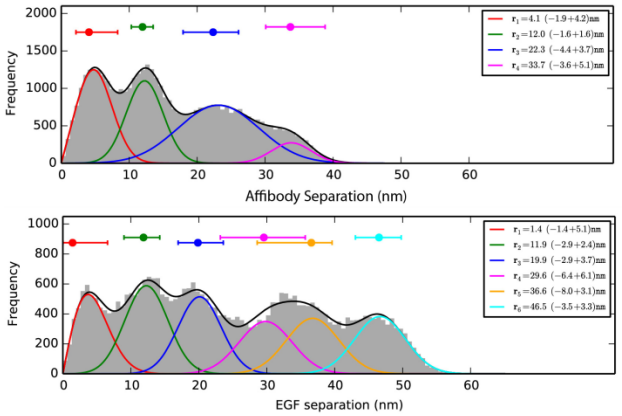

Figure 2. FLImP distribution. FLImP distribution (grey) of CF640R fluorophore-conjugated Affibody (top) or EGF (bottom) on CHO cells expressing ~10^5 copies of wild-type EGFR per cell. The Affibody plot contains data from 37 FLImP measurements and the EGF plot contains 30 FLImP measurements. The distribution is decomposed into a sum of four or six Rician peaks. Positions and error estimates are shown in the inset. The optimal number of peak components (colour lines) and the best-fit (black line) were determined using a Bayesian information criterion and Bayesian parameter estimation[6].

The FLImP distributions for EGF separations is compiled from those with confidence intervals of <4.8 nm. A Bayesian information criterion (see ref 6 for more details) determines the decomposition of the FLImP distribution of EGF separations into six Rician peak components. The peak in the range of 0–6.5 nm (Figure 2 bottom red peak) reflects separations comparable to or below the 4.8 nm resolution of the FLImP measurements; therefore the best-fit position of the peak can be susceptible to a bias. For this reason, we quote the confidence interval range and not the best-fit position. The separations in the other five peaks in Figure 2 bottom fall in the region free from such bias and their best-fit peak positions (11.9, 19.9, 29.6, 36.6 and 46.5 nm) reflect the underlying pairwise EGF separations. The 11.9 (-2.9 +2.4) nm peak likely represents two-ligand back-to-back dimers which give a separation of 12.5±0.3 nm. The larger peaks reflect the presence of higher order EGFR oligomers at physiological EGF concentrations. The presence of EGFR oligomers upon ligand binding have been previously reported[7,8]. The 19.9 (-2.9 +3.7) nm is consistent with that predicted for a tetramer[6] and the three other peaks are remarkable consistent with those of a hexamer, octamer and decamer[6].

The structure of inactive EGFR complexes.

As already mentioned, the FLImP distribution of 4 nM EGF exhibits a short peak in the range of separations of <6.5nm. Similar short EGF separations have been previously observed using FRET[7,9]. This is also observed in the FLImP distribution of CF640R fluorophore-conjugated anti-EGFR Affibody[10] which showed a peak in the range of separations of <8 nm (Figure 2 top). As the Affibody inhibits EGFR activity and competes with EGF for the same binding site[10,11], this peak likely reflects a set of complex structures of inactive receptors which persist despite the presence of EGF. The Affibody FLImP distribution also exhibits a second prominent peak at 12.0 ± 1.6 nm indicative of another form of inactive dimers.

Conclusions

We find that at physiological EGF concentrations, EGFR assembles into oligomers, as indicated by pairwise distances of receptor-bound fluorophore-conjugated EGF ligands. The pairwise ligand distances correspond well with predictions from structural models of EGFR dimers, tetramers, hexamers, octamers and decamers.

Acknowledgements

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