# Structure-function relationships and supra-molecular organisation of the Epidermal Growth Factor Receptor (EGFR) on the cell surface

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## Introduction

Oligomerization and clustering of protein complexes in the plasma membrane are an important feature of many biological processes, including cell signalling. The Epidermal Growth Factor receptor (EGFR) is one of the ErbB family of four receptor tyrosine kinases that initiate signalling cascades promoting cell proliferation, motility, and survival[1]. The extracellular domain of this receptor has a four sub-domain structure, and in the absence of its ligand is held in a closed or "tethered" conformation[2]. Upon ligand binding, the tethered structure opens into an extended form, which is capable of forming a dimer with another receptor[3]. Ligand-induced EGFR dimerization is believed to be the key step for the initiation of EGFR signalling, through the formation of an asymmetric dimer and allosteric transactivation of the two kinase domains. However, there is evidence that the situation in cells is significantly more complex than that described by the ligand-induced dimerization model. There have been a number of reports of the existence of dimers and higher order oligomers of EGFR in cells in the absence of bound ligand[4-9].

There is therefore a requirement for methods capable of characterising receptor conformation, oligomerization and clustering on the cell surface, and environment currently intractable for X-ray crystallography. The distance measurements required to adequately characterise these phenomena are challenging, ranging from molecular dimensions in the order of 1 to 20 nm, to plasma membrane organisation at the level of nanodomains and picket fences, from 20 to 100 nm[10]. A technique that has showed promise for inter- and intramolecular distance measurements on the required length scales is single fluorophore localisation. This technique relies on the fact that the position of a single emitter can be determined to high precision through fitting a profile to the diffraction-limited spot[11]. Given a sufficiently high signal-to-noise ratio (SNR), localisation can be achieved with a precision of better than 2 nm. Recently we have described a variant of existing single-fluorophore localisation techniques has been described, that uses Bayesian algorithms for single molecule spot detection and intensity tracking, correction for sample drift, and Monte-Carlo bootstrapping to determine errors in molecule localization[12]. This method, fluorophore localisation imaging with photobleaching (FLIP), produces more accurate errors, and measures distance distributions with confidence intervals, without the need for prior knowledge of the number of discrete fluorophore-fluorophore separations present in the sample. This technique has been used to provide new information on the spatial distribution of inactive and active EGFR molecules in cells.

## Methods

A detailed description of the methods can be found in Needham *et al.,* 2013[12]. Briefly, an anti-EGFR affibody was labeled with the fluorescent probe Atto 647N at its single cysteine residue. The affibody was then used to label T47D cells, which were imaged in the single molecule TIRF microscope at the CLF's *Octopus* facility[13]. Data were analysed by the FLIP method previously described[12], providing information on the separations between labeled EGFRs.

## Results

FLIP has been used to characterise the separations between EGFR molecules in T47D cells. This cell line expresses EGFR at physiological levels (~ 7-15 x $10^3$ copies per cell)[14,15], avoiding the possibility that oligomers are the result of receptor overexpression. For the investigation of EGFR in its basal state, cells were labelled with a fluorescent anti-EGFR Affibody antagonist shown not to cause receptor activation. The distribution of separations between inactive EGFR is shown in Fig. 1. This shows a range of separations with peaks at 8 nm, 22 nm, 37 nm, 46 nm, and 57 nm. This could be explained through the formation of a linear polymer with a repeat periodicity consistent with EGFR-EGFR separation in a dimer, with the shortest peak corresponding to the dimer observed crystallographically.

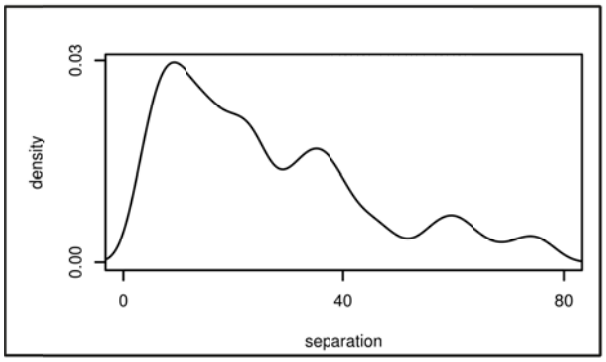


**Figure 1.** Separations of surface EGFR-Affibody complexes in T47D cells, measured using FLIP.

A model of this structure is shown in Fig. 2. These data appear to unequivocally demonstrate the existence of dimers and higher order EGFR oligomers in the basal state, and the low EGFR expression level in the cell line used rules out the possibility that this could be an over-expression artefact.

The authors speculate that the presence of the higher order oligomers might be due to the interaction of EGFR with actin filaments, for which there is prior evidence in the literature[16,17]. The separations measured would be consistent with receptor binding to cortical F-actin, which is a left-handed helix with a repeat of 35.9 nm[18]. We have recently obtained data that provide supporting evidence for this hypothesis; actin polymerization is disrupted by the depletion of plasma

membrane cholesterol, probably through interactions with phosphatidylinositol 4,5-bisphosphate (PIP2)[19].

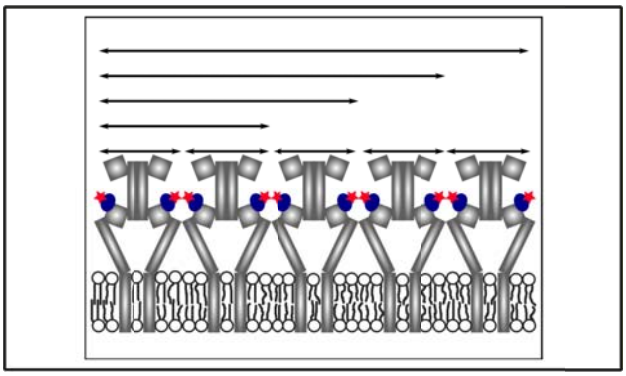

**Figure 2.** Model of EGFR homo-polymer consistent with the distances observed in Fig. 1.

Fig. 3 shows the distribution of EGFR separations in T47D cells following treatment with the cholesterol-depleting agent methyl-β-cyclodextrin. The 36 nm repeat suggested to be associated with cortical-F-actin binding is no longer present, compared with the data for untreated cells shown in Fig. 3A. A similar effect has been observed for GPI-anchored proteins, for which a mechanism of complex formation regulated by cortical actin activity has been suggested[20].

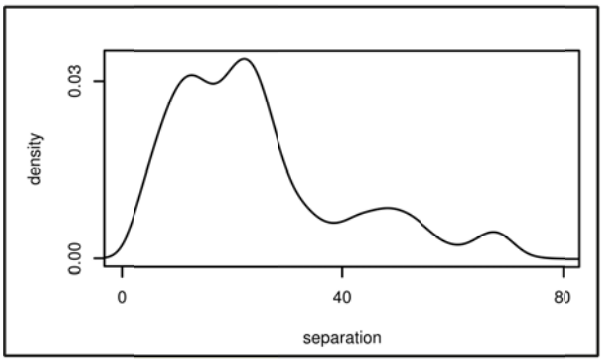


**Figure 3.** Separations of surface EGFR-Affibody complexes in T47D cells, measured using FLIP, following treatment with the cholesterol-depleting agent methyl-β-cyclodextrin, showing the loss of the ~36 nm repeat.

## Conclusions

Considerable advances have been made in the last decade in the understanding of EGFR dimerization and oligomerization in cells. These advances have been informed by high resolution structures but enabled by the development of new techniques for measuring in cells intermolecular distances ranging from 1 to 100 nm. In particular, single pair FRET techniques have been valuable for characterising short distances, and single molecule localization techniques vital for measuring the longer distances important in oligomer formation. The new FLIP technique developed at the CLF has been demonstrated to be an effective method for the measurement of distances in macromolecular complexes in cells. There now appears to be unequivocal data showing that EGFR dimers and higher order complexes exist in cells in the basal state, and that these complexes are not an artefact of receptor overexpression. Application of these new techniques should now begin to shed more light on the details of the polymerisation process, and therefore on EGFR activation of mechanisms.

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