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Human-EGFR aligned on the plasma membrane adopts key features of *Drosophila*-EGFR asymmetry

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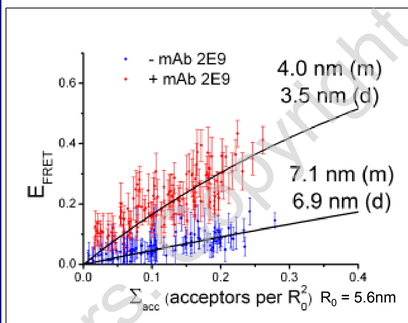
Summary

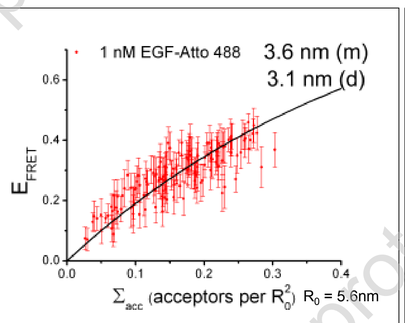
The ability of Epidermal Growth Factor Receptor (EGFR) to control cell fate is defined by its affinity for ligand. Current models suggest that ligand-binding heterogeneity arises from negative cooperativity in signalling receptor dimers, for which the asymmetry of the extracellular region of the *Drosophila* EGFR has recently provided a structural basis. However, no asymmetry is apparent in the isolated extracellular region of the human EGFR (hEGFR). Human EGFR also differs from the *Drosophila* EGFR in that negative cooperativity is only found in full length receptors in cells. To gain structural insights into the human EGFR *in situ* we developed an approach based on quantitative Förster resonance energy transfer (FRET) imaging, combined with Monte-Carlo and molecular dynamics simulations, to probe receptor conformation in epithelial cells. We experimentally demonstrate a high-affinity ligand-binding human EGFR conformation consistent with the extracellular region aligned flat on the plasma membrane. We explored the relevance of this conformation to ligand-binding heterogeneity and found that the asymmetry of this structure shares key features with that of the *Drosophila* EGFR, suggesting that the structural basis for negative cooperativity is conserved from invertebrates to humans, but in human EGFR, extracellular region asymmetry requires interactions with the plasma membrane.

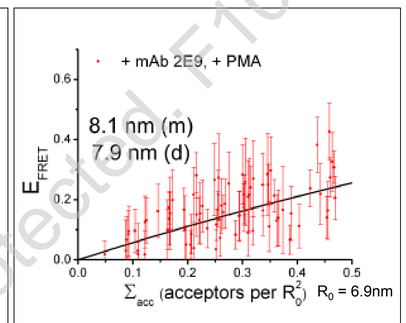
A short ligand-membrane distance of closest approach is found in hEGFR but not when high affinity EGF binding is abolished



Blue data points = 90-95% low affinity EGF binding sites occupied with labelled EGF

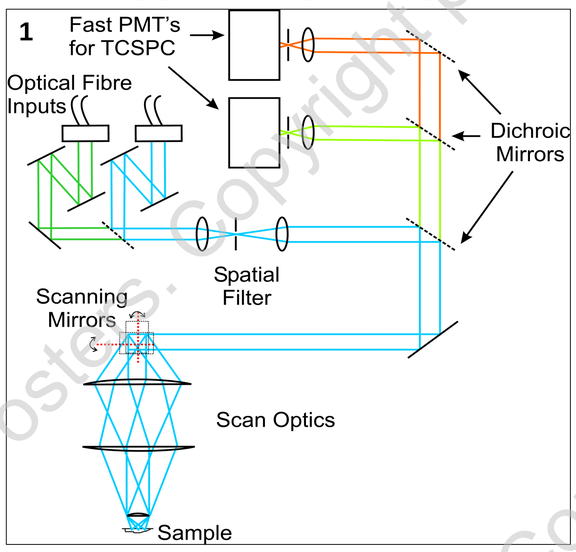
Red data points = ~48% high affinity EGF binding sites occupied with labelled EGF
The proportion of low affinity receptors able to bind to EGF is reduced by the use of an antibody (mAb 2E9) or by labelling cells with a lower concentration of EGF.

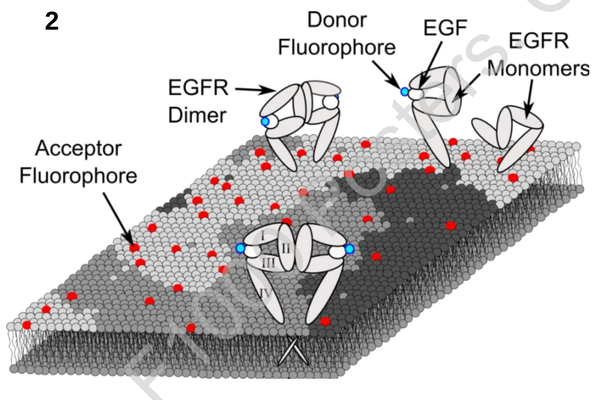




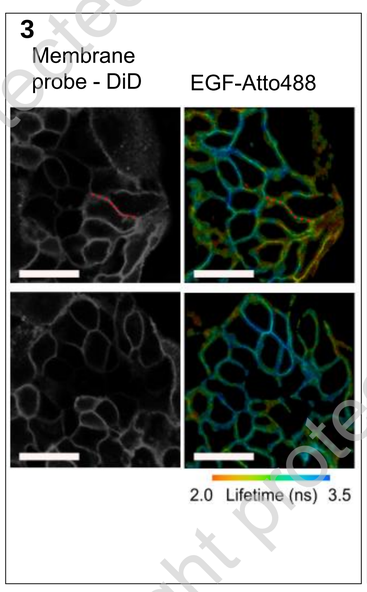
~10% EGF binding sites ONLY occupied with labelled EGF.
EGFRs phosphorylated intracellularly at Thr 654 by PMA, which abolishes high affinity EGF binding

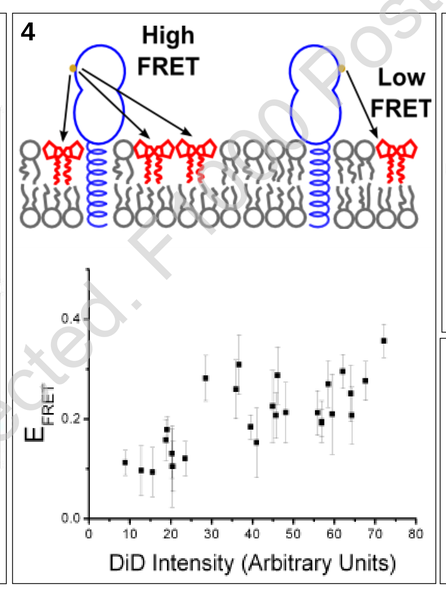
The application of a FRET-FLIM method to determine distances of closest approach (DOCA) for EGF/hEGFR complexes in A431 cells

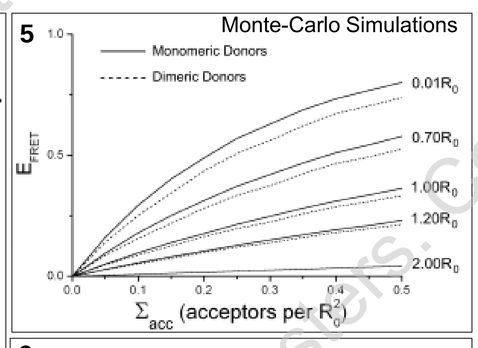


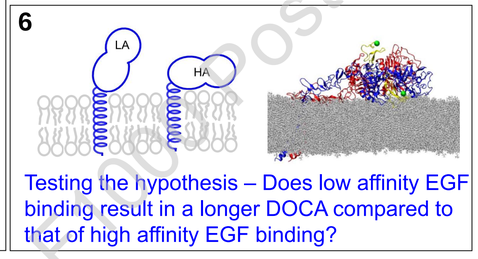


Confocal FLIM (**1**) can be used to detect FRET between donor and acceptor fluorophores (**2**) by measuring the lifetime quenching of the donor fluorescence lifetime in the presence of acceptor. Analysis of lifetime images of A431 cells labelled with Vybrant DiD and then challenged with EGF-Atto488 (**3**) can give a distance of closest approach (DOCA) of the EGF-binding site in human EGFR (hEGFR) to the membrane surface. This requires values for the efficiency of FRET over a range of acceptor density measurements (**4**) together with data analysis based on Monte-Carlo simulations (**5**, Corry et al. Biophys. J. 2005). We applied this quantitative method to follow up initial evidence for tilted and proud EGF/hEGFR complexes (Webb et al. Biophys. J. 2008). In addition, we investigated the relevance of an asymmetric model of the EGFR dimer, that has been shown by molecular dynamics simulations (Kästner et al. J.Struc. Biol. 2009), to EGF binding heterogeneity (i.e. high- and low-affinity binding kinetics) and negative cooperativity (**6**).



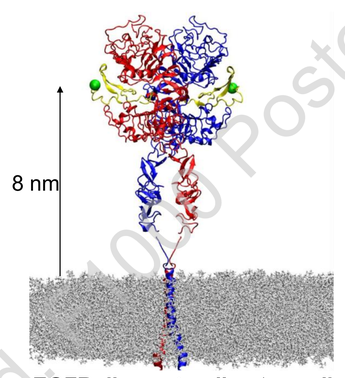




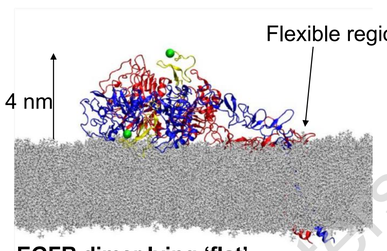


Testing the hypothesis – Does low affinity EGF binding result in a longer DOCA compared to that of high affinity EGF binding?

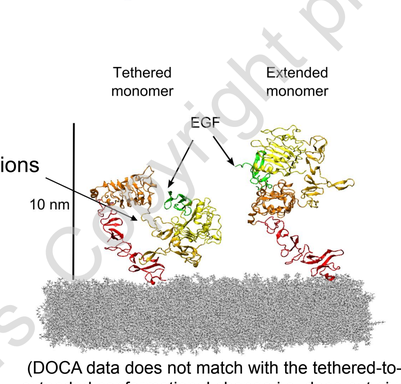
Molecular dynamics (MD) simulations/structures – Tilting a hEGFR dimer at a flexible region close to the membrane creates a new ectodomain conformation that is asymmetric…



EGFR dimer standing 'proud'



EGFR dimer lying 'flat'



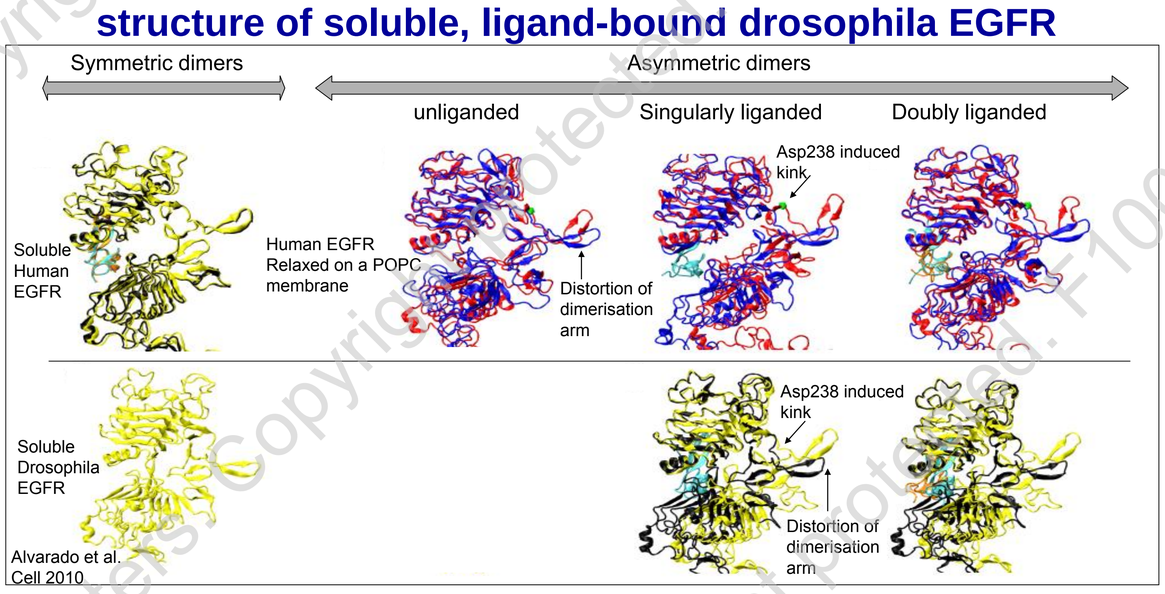
(DOCA data does not match with the tethered-to-extended conformational change i.e. does not give a large change in ligand-membrane separation)

…and the DOCA data in light of the MD simulations shows EGF binding heterogeneity occurs because hEGFRs aligned with the membrane create two distinct EGF binding sites.

Control experiments have shown that the 'proud' and 'flat' conformations also exist in –
- resting and stimulated cells – suggesting that the conformations are preformed and are independent of EGF ligand binding
- live cells - these hEGFR ectodomain structures do not occur because of fixation reagents
- cells depleted of cholesterol – release of receptors from the inhibitory effects of a lipid raft environment yields the same result (also control experiments show no differences in FRET efficiency when we used different lipid probes that partition into distinct microdomains of the cell membrane so it is unlikely that heterogeneous co-localisation of receptors and acceptors on sub-micron scales can explain the above results)
- HeLa cells – short ligand-membrane distances are present in cells that do not over-express hEGFR

Real-time FRET data in live cells show that a transient conformational change accompanies high affinity receptor signalling –
Another experimental approach shows that the distance from EGF (bound predominantly to high affinity binding sites) to the membrane decreases in a transient manner that correlates with an increase in Ca^{2+} signalling. The structures above are likely to represent two snapshots of dynamic receptor populations and receptors may sample a range of extracellular orientations.

Surprisingly the simulated asymmetric structure for membrane aligned hEGFR shares similarities with the crystal structure of soluble, ligand-bound drosophila EGFR

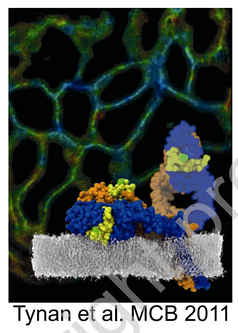


Discussion and future directions for study

Alvarado et al. argues that the observed asymmetry in doubly-liganded drosphila EGFR (dEGFR) explains the negative cooperativity seen in soluble dEGFR ectodomains and provides a structural basis for high and low affinity sites. We suggest that our MD simulations show that the alignment of hEGFR ectodomains on a membrane surface is sufficient to introduce an asymmetry to receptor dimers that is very similar to that observed in soluble dEGFR ectodomains; suggesting that with the aid of the membrane negative cooperativity could be achieved via asymmetry in hEGFR. We therefore propose that the structural basis for negative cooperativity is conserved from invertebrates to humans.

Human EGFR intracellular interactions must also be involved in promoting the flat configuration as hEGFR mutants with deleted intracellular domains do not show negative cooperativity.

Our MD simulations illustrate an ability of hEGFR extracellular subdomains to reorient themselves according to the presence or absence of ligand and according to the environment. We speculate that such flexibility may also be important if members of the mammalian EGFR family are to have the ability to form several heterodimers with other members of the family.



Tynan et al. MCB 2011

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