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Investigating the Conformation of HER Membrane Proteins in Cells via Single Molecule and FLIM Microscopy

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1. Introduction

Understanding how signal inputs and outputs are organised in membrane protein signalling networks is an important question in biology. The current goal is to derive methods that would allow the 'watching' of these network proteins in action and at atomic resolution to see details of their structure. This requires the addition of a 'time' dimension to structural biology so that the spatio-temporal parameters of all atoms in each protein can be described in detail. This is a huge challenge that in cell-free systems has begun to be partially addressed through dynamic experiments combined with molecular simulations. However, in cells, the functions of particular structural motifs are not just constrained by Brownian motions, energy landscapes and thermodynamics, but also by the local availability of partners in subcellular compartments and the boundary constraints imposed by cell environments, for example in the plasma membrane, with its 2D dimensionality, local curvature and electric fields. To understand protein function in cells, observations have to be made in the only physiologically-relevant 'laboratory', the cell. This adds levels of complexity to an already vast challenge.

Using molecular biology techniques in combination with optical methods, we can now annotate individual genes and gene products, screen for protein-protein, protein-DNA and small molecule interactions, and quantify dynamic changes. However, only the combination of fluorescence imaging, fluorescence resonance energy transfer (FRET) and single molecule detection currently offers sensitive spatio-temporal detection in cells for low abundance protein interactions. This is beginning to bridge the gap between protein structure and function by allowing real-time quantitative observations of structural details,

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conformational intermediates, association and dissociation constants, diffusion rates, and rare events. Previous information on complex protein networks, such as the human epithelial growth factor receptor (HER) bio-system, has been derived generally from high-throughput screens and/or single cell models using ensemble (averaged) technologies such as biochemical extraction followed by mass spectrometric analysis. Here we describe examples of how single molecule and ensemble fluorescence microscopy methods can offer the means to understand and predict in cells the structure-function relationships of proteins in the input layer of the HER signalling network, from the changes in complex interactions between their microscopic molecular components to their response to perturbations.

2. The human epidermal growth factor receptor (HER) family

HER molecules are prototypical examples of the growth factor receptor tyrosine kinase (RTK) super-family, which also comprises 18 sub-groups of cell surface receptors for many growth factors, cytokines and hormones (Schlessinger 2000). The HER family consists of four homologous receptors, known in cells of human origin as HER1, HER2, HER3 and HER4 (Citri & Yarden 2006). The HER1 molecule (also known as Epidermal Growth Factor Receptor (EGFR)) is the founding member of the RTK family (Fig. 1). In other mammals HER molecules are known as ErbB receptors (ErbB1-4). This name originates from an oncogenic erythroblastosis retrovirus (v-erbB) which encodes a mutated homologue of HER1 (Downward et al. 1984).

HER molecules are encoded as single pass trans-membrane proteins (Fig. 1). The primary structure of HER1, which is shared with all receptor tyrosine kinases, consists of a single polypeptide chain (1,186 amino acids) of 170 kD, containing a heavily glycosylated 622-amino acid residue amino-terminal extracellular ligand binding domain that is connected to the cytoplasmic domain by a single transmembrane (TM) helix of 23 residues. The 542-residue cytoplasmic domain contains a conserved 250-amino acid tyrosine kinase core (Ullrich et al. 1984). The kinase domain is the locus of the enzymatic activity of the receptor and at the core of its signalling function in the cell.

Mature HER molecules are translocated to the plasma membrane lipid bilayer, which is the outer boundary of the cell separating the extracellular and intracellular environments (Hillier & Hoffman 1953), were they are able to accept signalling cues from their environment (Fig. 1). In the HER family signalling cues are provided by 13 potential ligands (reviewed in Yarden & Sliwkowski 2001; Citri et al. 2003; Citri & Yarden 2006). These ligands, known as epidermal growth factor (EGF)-related peptides, can be classified in three functional groups according to their specific binding targets (Olayioye et al. 2000): EGF, transforming growth factor alpha (TGFα), amphiregulin and epigen bind HER1; betacellulin, epiregulin and heparin binding EGF-like growth factor bind HER1 and HER4; neuregulins 1 to 4 (NRG, also known as hereregulins), bind either both HER3 and HER4 (NRG-1 and NRG-2) or HER4 alone (NRG-3 and NRG-4). HER2 is thought to be an orphan receptor, with none of the EGF family of ligands discovered so far being able to bind and activate it (Baselga & Swain 2009; Olayioye et al. 2000).

EGF-related peptide growth factors are synthesised as cell membrane associated precursors in the plasma membrane. The extracellular domains of membrane-bound growth factors are subsequently shed via the action of members of a family of proteases known as a disintegrin
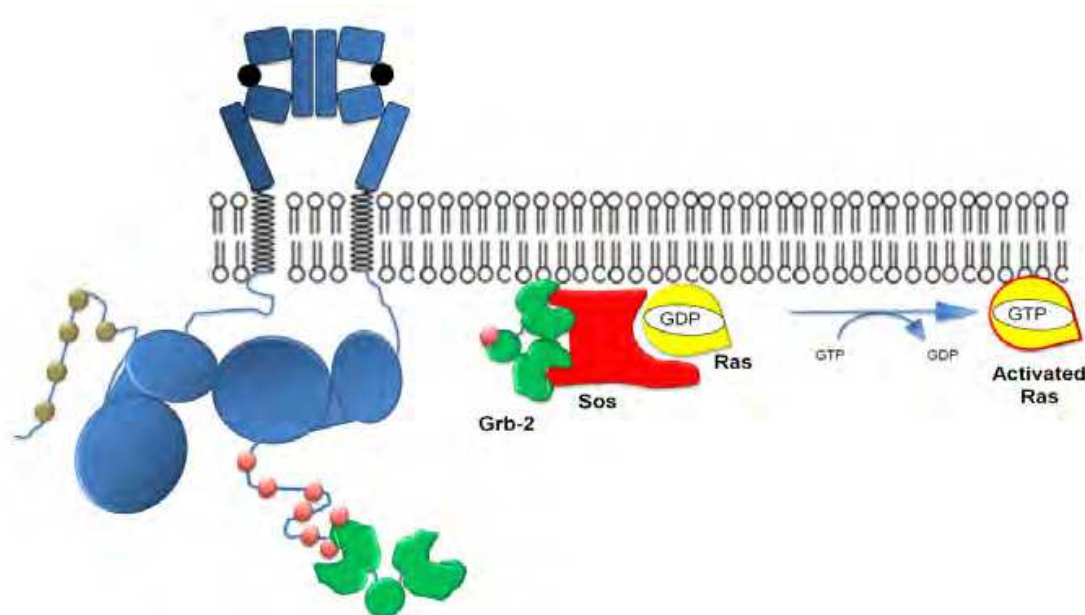
Fig. 1. A cartoon of a 2:2 EGF/HER1 dimer complex showing the initial stages of effector recruitment and signalling. Shown are the external domains of HER1 bound to EGF (black spheres) derived from crystallographic data (Garret et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003). In the cytoplasm the two tyrosine kinase domains of the HER1 dimer form an asymmetric dimer (Zhang et al. 2006). Following recruitment and phosphorylation of effectors like Grb2, recruitment of Sos leads to the activation of Ras via exchange of GTP for GDP to activate the Ras mediated signalling pathway (Zhang & Liu 2002).

and metalloproteases (ADAMs) family. As indicated by their name, these proteolytic enzymes are zinc-dependent trans-membrane metalloproteases (Zhou et al. 2005). Other members of the ADAMs family also shed extracellular domains of other cytokines and receptors (Edwards et al. 2008). Growth factor shedding is critical for the production of soluble functional HER ligands that can activate cell signalling via paracrine and autocrine mechanisms. Growth factors that remain membrane-bound can bind HER molecules on adjacent cells leading to juxtacrine signalling (Riese & Stern 1998).

3. Ligand-induced receptor dimerisation

An essential step in HER activation and signalling is achieved via ligand-induced receptor dimerisation (Schlessinger 2000). Ligand molecules bind the extracellular region of their cognate receptor in specific sites promoting dimerisation and interactions between two receptor monomers (Fig. 1). If two receptors are of the same type (e.g. HER1-HER1 dimer) the process is known as homo-dimerisation. For receptors of two different types (e.g. HER1-HER3) the process is known as hetero-dimerisation. Inactive receptors are believed to exist mainly as monomers, although unliganded (inactive) HER dimers have also been detected in cells (Martin-Fernandez et al. 2002; Clayton et al. 2005).

A defining characteristic of the HER family is that only HER1 and HER4 can bind ligands and also signal autonomously via homo-dimerisation and trans-activation of their tyrosine kinases (Yarden & Sliwkowski, 2001). In contrast, HER2 and HER3 are not autonomous. As

discussed above, HER2 lacks the intrinsic ability to interact with known ligands, whereas the kinase of HER3 is defective (reviewed in Citri et al. 2003). HER2 and HER3 can therefore only initiate signals through hetero-dimer formation. The following interacting pairs have been reported in the literature: HER1-HER1, HER4-HER4, HER1-HER2, HER1-HER3, HER1-HER4, HER2-HER3, HER2-HER4 and HER3-HER4 (reviewed in (Bublil & Yarden 2007)). HER2-HER2 and HER3-HER3 homo-dimers may also exist, but this is less certain. Despite having no soluble ligand, HER2 is important because it is the preferred hetero-dimerisation partner of the other ligand-bound family members (GrausPorta et al. 1997). In addition, there are also reports of higher order homo-oligomers of HER1 (such as tetramers) (Clayton, et al. 2005; Clayton et al. 2008).

4. HER signalling in cancer

Aberrant behaviour of HER family members has been implicated in many cancers (Sharma et al. 2007). Research has shown that in adulthood, excessive HER signalling upsets the balance between cell growth and apoptosis resulting in the development of a wide variety of solid tumours (reviewed in Bublil & Yarden 2007). In particular, the level of expression and/or activation of HER1 and HER2 are altered in many tumours of epithelial origin, and clinical studies indicate that HER1 and HER2 have important roles in tumour aetiology and progression (Hynes & Lane 2005). For example, deregulated signalling by cell surface HER1 receptors (e.g. via activating mutations in the *HER1* gene) is implicated in a substantial percentage of lung cancers (Paez et al. 2004). Gene amplification leading to HER1 overexpression is also often found in other human cancers like glioblastoma and esophageal squamous cell carcinoma (Ohgaki et al. 2004; Sunpaweravong et al. 2005). As activation of HER molecules has been shown to result in the growth and progression of the malignancy, the HER family is an important target of the pharmaceutical industry and there have been considerable research efforts directed toward the development of effective inhibitors of HER signalling. Two important types of HER inhibitor are in clinical use: Humanized antibodies directed against the extracellular domain of HER1 or HER2, which elicit an immune response and/or block ligand-binding and dimerisation, and small-molecule tyrosine-kinase inhibitors (TKIs) that compete with ATP in the tyrosine-kinase domain of the receptor inhibiting its intrinsic tyrosine kinase activity (Hynes & Lane 2005). Some of these TKIs have already demonstrated substantial clinical activity against several cancers (e.g. targeting of HER1 and HER2 are in different stages of pre-clinical and clinical trials) (Bublil & Yarden 2007; Citri & Yarden 2006; Hynes & Lane 2005).

5. Structural insights on the HER family: The extracellular domain

The original paradigm proposed for the activation of HER molecules was that the binding of ligand induced the dimerisation of monomeric unliganded receptors via ligand-crosslinking of two receptor moieties (Schlessinger 2000). Contrary to these expectations, crystallographic studies of HER1 ectodomain fragments bound to two types of ligands, EGF and TGFα, have shown quasi-symmetric 2:2 ligand-receptor dimers where each ligand binds simultaneously to subdomains I and III of one of the monomers (Fig. 2). These HER1 dimer structures therefore clearly showed that the dimerisation of this receptor was not directly mediated by the binding of ligand but achieved exclusively via receptor-receptor contacts via subdomain II (Garrett et al. 2002; Ogiso et al. 2002) (Fig. 2).

Crystal structures of unliganded HER1, HER3 and HER4 monomers show that these are held in a closed conformation by an intramolecular tether formed by loops in subdomains II and IV (Bouyain et al. 2005; Ferguson et al. 2003; Hyun-Soo & Leahy 2002). These data suggest that ligand binding and dimerisation involves major extracellular structural rearrangements in HER1, HER3 and HER4 molecules because in ligand-occupied receptor dimers the intramolecular tether is broken and the receptor is opened into an extended conformation which interacts with another monomer to form a back-to-back dimer (Burgess et al. 2003; Ferguson, et al. 2003). Interestingly, unliganded HER2 has an extended configuration that resembles the structure of ligand-bound 'activated' HER1 (Cho et al. 2003). This may explain the unique properties of HER2, which has no known ligand and can cause cell transformation (and tumorigenesis) by simple overexpression (Yu & Hung 2000). The latter appears to force the equilibrium towards spontaneous HER2 homodimer formation, which leads to receptor activation in the absence of ligands. This is the situation observed in a variety of human cancers.
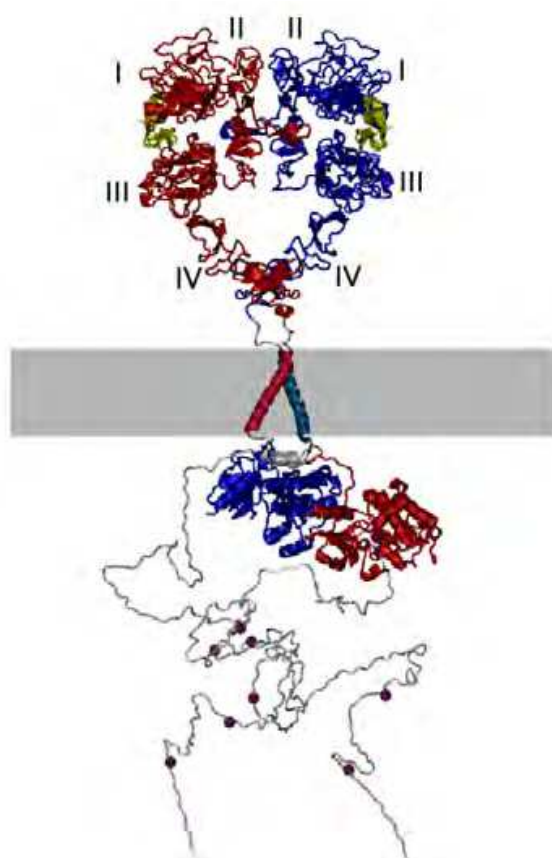

Fig. 2. A structural model of the 2:2 EGF/HER1 complex. Shown are the four subdomains (I–IV) of the HER1 ectodomain (Garret et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003) and the TM helices. In the cytoplasm, the two tyrosine kinase domains form an asymmetric dimer (Zhang et al. 2006). The EGF ligands are shown in yellow. Fragments known from crystallography or NMR are coloured, while the pale grey sections denote regions of uncertain structure, including the extracellular JM linker, the intracellular JM domain (Jura et al. 2009a; Brewer et al. 2009), and the C-terminal tails. On the latter, purple spheres indicate known auto-phosphorylation sites. Figures have been prepared using visual molecular dynamics (Humphrey el al. 1996).

The typical high-resolution representation of ligand-bound HER1 dimeric holoreceptors is depicted in Fig. 2. This generally accepted model has been derived by putting together the available structural information on extracellular and intracellular fragments (Garrett et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003; Brewer et al. 2009; Jura et al. 2009a; Jura et al. 2009b; Zhang et al. 2006). In this model, the extracellular domains are oriented with respect to the membrane based on the historical view that receptors are protruding from the plasma membrane as antennae.

6. Structural insights into the HER family: The intracellular domain

Ligand binding and the dimerisation of HER1 extracellular domain leads across the plasma membrane to the formation of an asymmetric dimer by the two associated intracellular kinases (Zhang et al. 2006) that is stabilised by the inner juxtamembrane (JM) region (Brewer et al. 2009; Jura et al. 2009a) (Fig. 2). Kinase activation follows through an allosteric mechanism in which the C-lobe of one kinase (activator) "pushes" the N-lobe of the other kinase (receiver) in an interaction that is highly reminiscent of the activation of CDK2 by binding of cyclin A (Jeffrey et al. 1995). Active kinases in the homodimer (and possibly heterodimer) then proceed to phosphorylate *in trans* tyrosine residues in the C-terminal tail of the partner receptor (Zhang et al. 2006). Fig. 2 depicts the C-termini of a HER1-HER1 dimer (for which there is no structural data), showing the location of tyrosine amino acid residues. These tyrosine residues are phosphorylated by the addition of a phosphate group (PO_4^{3-}). When phosphorylated these sites act as docking sites for a large repertoire of intracellular adaptors and enzymes containing Src homology 2 (SH2) domains (Pawson 2004). These include Grb2 (Fig. 1), GAP, Shc (Pai & Tarnawski 1998), phospholipase Cγ (PLCγ) (Katan 1998), and phosphatidylinositol 3'-kinase (PI3K) (Vanhaesebroeck et al. 2001), which regulate Ras/Rho-like GTPases (Ridley 2001), Ca^{2+} second messenger production (Defize et al. 1989), and the Ras-activated MAP/SAP kinase pathways (Zhang & Liu 2002). In this way, HER molecules start the intracellular signalling cascades that lead to cell proliferation, changes in cell morphology, trafficking, and the termination of signals via endocytosis of the receptor-ligand complexes (Carpenter 2000; Sorkin 2001).

The pattern of phosphorylated tyrosine motifs is specific to each HER homo and hetero pair (Schulze, Deng, & Mann 2005a). This is the way by which intracellular effector proteins can selectively be recruited by different HER pairs at the plasma membrane to initiate different signalling events and cascades in the cell (reviewed in (Citri & Yarden 2006)). The combinatorial variety of homo/hetero HER1-4 complexes therefore provides a mechanism by which different cell responses can be induced via recruitment of different combinations of signalling effectors to phosphorylated homo and hetero HER complexes, activating intracellular pathways for signal attenuation (receptor desensitisation, down-regulation, endocytosis and trafficking), signal amplification and cell growth, death signals (apoptosis) and other signal processing pathways (Yarden & Sliwkowski 2001). This may explain how subtle differences in the expression pattern of HER1-4 and their ligands may ultimately regulate development and homeostasis in many tissue types.

7. Towards a systems biology perspective

A glimpse of the true complexity in the HER network has only been obtained recently in the light of high-throughput screens and proteomic assays. These have added hundreds of

putatively assigned new interactions and several new signal regulators in different cellular compartments to an already complicated picture, making the network increasingly difficult to describe. The burgeoning of data is facilitating the emergence of a cellular systems level perspective (Jones et al. 2006; Schulze, Deng, & Mann 2005b) which is beginning to uncover non-intuitive features of HER signalling, such as the high biological potency of a low-affinity mutant of EGF (Schoeberl et al. 2002). The emerging view is that of a complex 'fail-safe' robust network system (Citri & Yarden 2006), which is able to maintain some degree of function through embedded layers of functional degeneracy, modularity, redundancy, buffering and signalling controls, even if some components are damaged. The robustness of the HER signalling network is underlined by its bi-stability (ligand-regulated ON/OFF states) and its 'bow-tie' configuration, in which multiple positive cues (ligand binding) and negative cues (receptor trafficking) are fanned out through an intermediate core processing unit. The core consists of multiple isoforms of interconnected effector units and signalling cascades, intertwined with densely coupled subnetworks (e.g. the phosphoinositide and Ca^{2+} networks and the endocytic machinery; see (Sorkin 2001)). The number of intracellular proteins that form part of this core is vast, including not only the HER1 effectors outlined above, but also entire families of adaptors and enzymes of (e.g. Grb2 and Shc) (Pai & Tarnawski 1998), ubiquitin ligases (e.g. the E3 ubiquitin ligase c-Cb) (reviewed (Thien & Langdon 2005)), lipid and protein kinases (e.g. PI3K, Akt; reviewed (Vanhaesebroeck, et al. 2001)), GTPases (Ridley 2001) (e.g. Ras, Rho) and phospholipases (e.g. PLC-γ (Katan 1998)). More recently, the activation of the small RhoGTPase Cdc42 by HER1 signalling has also been established (El-Sibai et al. 2007; Feng et al. 2006; Kurokawa et al. 2004). HER signalling also results in the specific induction of outputs through transcription factors (Grandis et al. 1998) (e.g. STAT3) which, depending on the exact combination recruited through the core and the cellular context, ultimately lead to different cell fates, including proliferation, survival, adhesion, migration and differentiation (Oda et al. 2005).

8. Multi-colour labelling of signalling proteins in the HER network

Because of the complexity of the HER signalling network (Oda, et al. 2005), the systems-level perspective provides a useful framework to describe network behaviour and to expose and predict the fragility of network activity centres. However, the bottleneck in mathematical 'Systems' modelling is incomplete biological understanding. This is mainly due to the difficulties of obtaining quantitative kinetic/dynamic data and structure-function relationships in the cellular environment. To gain a better understanding of HER signalling that can facilitate a systems perspective it is necessary, for example, to devise better means of obtaining structural data on the input layer of the HER signalling network. It is also necessary to obtain much more information on the dynamic rules of engagement between HER network proteins in vivo and to understand the positive and negative feedback-loops regulating the HER network that allow functioning under perturbation.

Fluorescence microscopy methods provide a convenient approach to study protein conformation and dynamic interactions between HER network members at the plasma membrane of living cells. This method is non-invasive and allows several types of proteins to be specifically tagged and observed simultaneously. For example, heteromeric pan-HER interactions can in principle be measured in living cells by using a combination of fluorophores to label each of the four members of the HER family with a different colour tag

(Fig. 3) and then employing FRET and/or multicolour single molecule tracking to follow their interactions in real-time (see below). These methods can in principle be extended in cells bio-engineered to express specific combinations of interacting fluorescent ligand/HER1-4/adaptor/inhibitor variants.
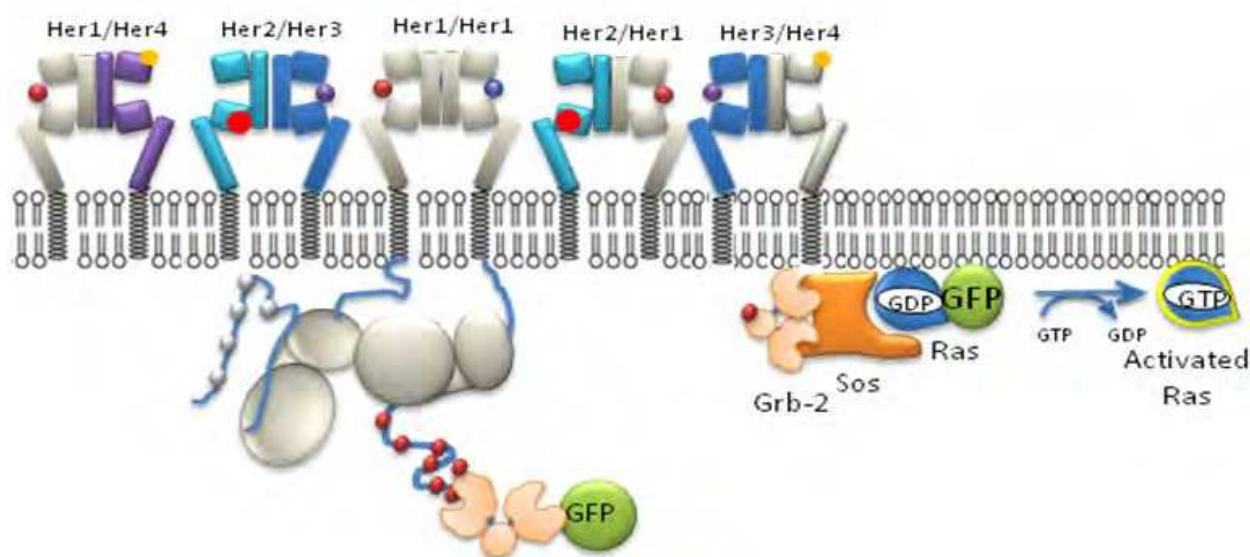

Fig. 3. A cartoon showing the HER1 homo-dimer and hetero-dimers at the plasma membrane. (The intracellular domains of hetero-dimers are not shown for simplicity). Different colour labels are targeted to the extracellular domains (small colour spheres). Intracellular proteins can be labelled using fluorescence proteins such as GFP.

One strategy to label HER molecules is to use their cognate ligands (e.g. EGF or small affibodies) as delivery vehicles for the fluorophores (Fig. 3). For this, fluorophores of different colours can be bound in a 1:1 ratio to different activating and non-activating ligands, each specific for one of the receptor types. Once these ligands bind their cognate receptors, each receptor type will be tagged with a different colour molecule. This means that each receptor type is colour-coded and will appear in a different colour channel. Other strategies include chemical synthesis of fluorescent ACP- (a 6 kDa, acyl carrier protein), MCP- (a mutant version of ACP), SNAP- and CLIP-tag substrates, fluorescence protein fusions to HER C-termini, N/C-termini of scaffolds and effectors (Fig. 2) and cell permeant benzylguanine (BG) or benzylcytosine (BC) dye derivatives (Banala, Arnold, & Johnsson 2008), including BG and BC conjugated to TMRstar (which can fluorescently label any O6-alkylguanine-DNA alkyltransferase (AGT)-tagged, i.e. SNAP or CLIP tagged, intracellular proteins in live cells).

The remainder of this chapter shows steps taken in our laboratory towards the development of an integrated experimental approach that can provide data on multi-molecular interactions towards a system-focused approach. These steps have so far consisted of developing the tools to extract structural and dynamic information on the HER bio-system in the cellular context by gathering information on conformational changes, receptor interactions and the dynamic rules of engagement in HER signalling.

9. Investigating HER protein-protein interactions by FRET

FRET is a phenomenon by which the excited-state energy of an optically excited fluorescent molecule (donor) is transferred to a neighbouring fluorescent molecule (acceptor) non-radiatively via intermolecular Van der Waals (dipole-dipole) interactions (Stryer & Haugland 1967). For FRET to occur the electronic levels of donors and acceptors must overlap. FRET depends on the distance between donors and acceptors as the inverse of the sixth power and is therefore very sensitive to short inter-molecular distances in the range ~2-8 nm. It can therefore be a useful tool to investigate molecular interactions.

FRET observations can be made either in the steady-state, by detecting the quenching of the fluorescence emitted by the donor as energy is transferred to the acceptor, or time-resolved, by measuring the shortening of the fluorescence lifetime of the donor. FRET imaging between fluorescent EGF derivatives bound to HER1 molecules in cells has been widely employed to investigate HER1 homo-dimerisation (Gadella & Jovin 1995; Sako et al. 2000; Martin-Fernandez et al. 2002; Clayton et al. 2005; Clayton et al. 2008). A popular strategy for this was to measure the efficiency of FRET between donor and acceptor fluorophores bound to the N-termini of receptor-bound EGF ligands. Although distances shorter than ~8 nm (averaged over the receptor population) were ubiquitously reported by FRET in these conditions in cells, it should be noted that the preferred (back-to-back) crystallographic dimer shows N-terminal inter-ligand distances of >11 nm (Fig. 3). This distance is too large to be detected by FRET and will remain so even when extreme deformations are applied to this crystal structure; for example, by changing the angle between domains I and III and domain II and/or applying extreme perturbations along low frequency normal modes. The FRET results therefore suggest that other interfaces between receptor monomers not yet found by crystallographic methods must also occur at the plasma membrane of cells.
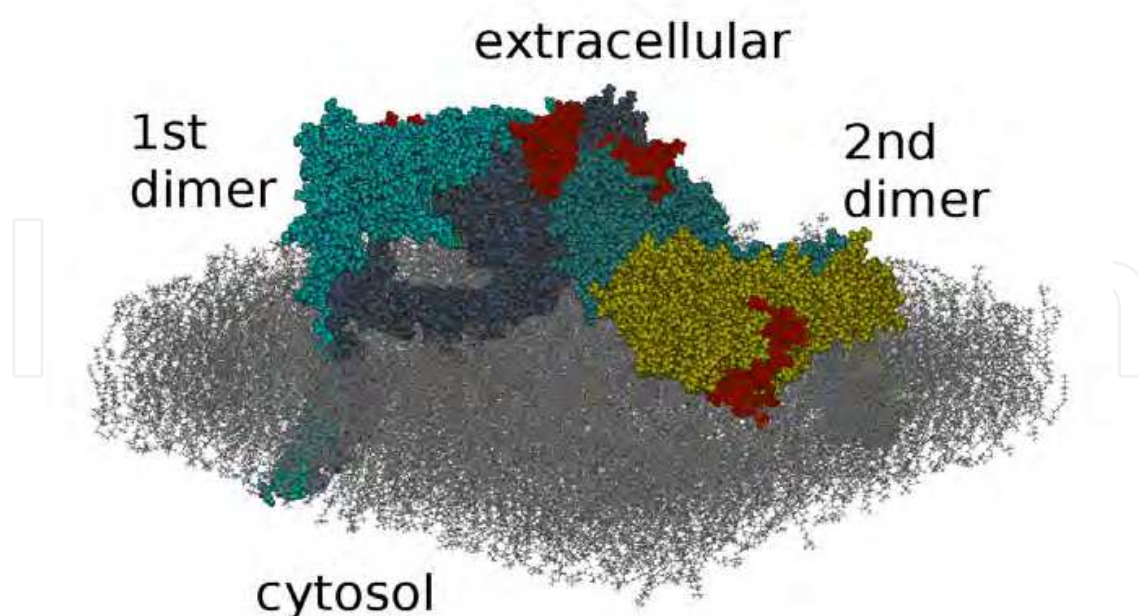

Fig. 4. The back-to-back / head-to-head / back-to-back tetramer on the plasma membrane (Kästner et al. 2009). The grey- stick models are the atomistically modelled lipid molecules of the membrane. EGF ligands are shown in red.

Attempts to understand the origin of FRET between donor/acceptor EGF ligands bound to HER1 include the pioneering work by Clayton et al. in intact BaF/3 cells, a murine interleukin-3 dependent pro-B cell line which do not overexpress ErbB1 (the mammal HER1 equivalent). Using fluorescence correlation microscopy, this work showed the presence in cells of tetramers of activated cell surface EGF/ErbB1 complexes. FRET in turn showed the presence of very short distances (< 4 nm) between ErbB1-bound EGF ligands (Clayton, et al. 2005). It was postulated that these short distances could explain the presence of FRET in the context of a tetramer formed by two back-to-back crystallographic dimers. These short distances between EGF ligands bound to HER1 were subsequently found in human A431 epithelial cells by single pair FRET imaging (Webb et al. 2008). This work provided additional evidence for HER1-HER1 interfaces in cells other than that described in the back-to-back crystallographic structure.

The work of Clayton et al. 2005 and Webb et al. 2008 set the basis for two new models of HER1 activation: In one model tetramers were generated through side-by-side contacts between two adjacent back-to-back dimers; however, there is no crystallographic evidence yet for this arrangement. In the second model (Fig. 4) tetramers were made of two back-to-back dimers joined by a weak, asymmetric "head-to-head" interface seen in crystal structures (Garret et al. 2002). This interface also results in short distances between the N-termini of the two bound EGF molecules of < 4 nm. Molecular dynamics (MD) simulations showed that the head-to-head interaction stabilises appreciably when the tetramer is relaxed on the membrane (its interface area increased from 443 $Å^2$ seen in the crystal structure to 604 $Å^2$) (Kastner, et al. 2009). In this tetramer the two dimers remained stable but lost the approximate 2-fold symmetry of the crystal structure. The average dimerisation interface area rose from 1,197 $Å^2$ to 1,483 $Å^2$, largely as a result of additional interactions between the N-terminus and the dimerisation arm, while the two ligands buried 1,166 $Å^2$ and 1,211 $Å^2$ at distinct binding sites.

Interestingly, the ligand-membrane distances predicted by the tetramer in Fig. 4 are significantly shorter than those predicted by the crystallographic back-to-back dimers standing upright from the membrane (Fig. 3), a hypothesis that can be tested using FRET (see section 11). Initial measurements by Webb et al. (2008) were consistent with the short ligand-membrane distances proposed by the model in Fig. 4 (see section 11).

10. Investigating the origin of ligand – Binding heterogeneity to HER1

Possibly the main disappointment of the HER crystal structures is that they did not explain a 30-year old puzzle in the HER signalling field, namely the origin of the characteristic heterogeneity in EGF-binding affinity to cell-surface HER1 molecules. This heterogeneity was first detected in EGF binding kinetic experiments in cells, as these display characteristic concave-up Scatchard plots (Magun et al. 1980; Shoyab et al. 1979). As HER1 is expressed as a single translation product (Ullrich & Schlessinger 1990), the concave-up EGF-binding Scatchard plots were interpreted as indicating the presence of two receptor populations: a small minority of high-affinity receptors with dissociation constants (K_D) of <1 nM that mediate most signalling events, and a majority of low affinity receptors with K_D of >1 nM (Defize et al. 1989; Friedman et al. 1984). When crystallographic structural data became available ~ 10 years ago, the high- and low-affinity populations were initially expected to be extended HER1 dimers and tethered HER1 monomers, respectively; however, in this model

stabilization of the extended dimer configuration by one ligand molecule would facilitate the binding of a second ligand to the remaining unliganded receptor in the dimer, resulting in positive cooperativity and therefore concave-down Scatchard plots (Mattoon et al. 2004).

Using global modelling of ligand-binding data as a function of receptor number, Macdonald and Pike subsequently showed that EGF-binding heterogeneity could be accounted for by negative cooperativity in HER1 dimers (Macdonald & Pike 2008). However, negative cooperativity requires that ligand binding to one subunit of the HER1 dimer decreases the ligand affinity of the other subunit. This would, in turn, require the interactions between ligand and the two subunits of the receptor dimer to be asymmetric, inconsistent with the symmetry observed in the back-to-back structure of HER1. A series of recent crystallographic structures recently showed that in the drosophila counterpart of HER1 (known as drosophila EGFR or dEGFR) the extracellular domain asymmetry is induced by the binding of the first ligand growth factor to the dEGFR dimer, which structurally restrains the unoccupied binding site, reducing the affinity for binding of the second ligand (Alvarado et al. 2010). Interestingly, concave-up Scatchard plots were observed in preparations of the isolated extracellular region of the dEGFR, in direct contrast with its human counterpart, in which concave-up plots are observed only when ligands bind to full-length receptors in cells. These differences suggest that other receptor regions, conformations, and/or other unknown cellular components must be involved in the regulation of ligand affinity in HER1 in the cellular environment. A better understanding of the conformation of HER1 in the plasma membrane environment is therefore required to understand the origin of the heterogeneity of EGF-binding.

11. Using FRET microscopy to report *in situ* protein conformation

Besides ligand-ligand interactions, FRET can also be employed to investigate the conformation of proteins at the plasma membrane, for example by measuring the distance from a specific site in the protein of interest (e.g. the binding site of a growth factor ligand) to the cell surface (Fig. 5). Protein-membrane distances can be determined from the variation of the efficiency of FRET between fluorescent donors attached to the protein of interest and acceptors labelling the cell surface, measured as a function of acceptor surface density (Fig. 5). When crystal structures of the protein and/or protein fragments are available, protein-membrane distances derived from FRET can be used to constrain the disposition of protein structures with respect to the plasma membrane to inform on protein conformation *in situ*.

Analytical expressions describing the energy transfer process between random distributions of donors (e.g. in specific sites in proteins) and acceptors on lipid membrane surfaces have been derived for a number of geometries (see for example Wolber & Hudson 1979). The solutions to these equations are the distance of closest approach between the protein-bound fluorescent donor probe and a lipid acceptor chromophore at the plasma membrane. Examples using this FRET method include an investigation to derive the mean distance between the EGF binding site of HER1 and the plasma membrane of cells in suspension (Carraway et al. 1990), changes in conformation of α4-Integrin during activation (Chigaev et al. 2003), and the minimum separation between the protein portion of GPI-anchored proteins to the bilayer surface (Lehto & Sharom 2002).
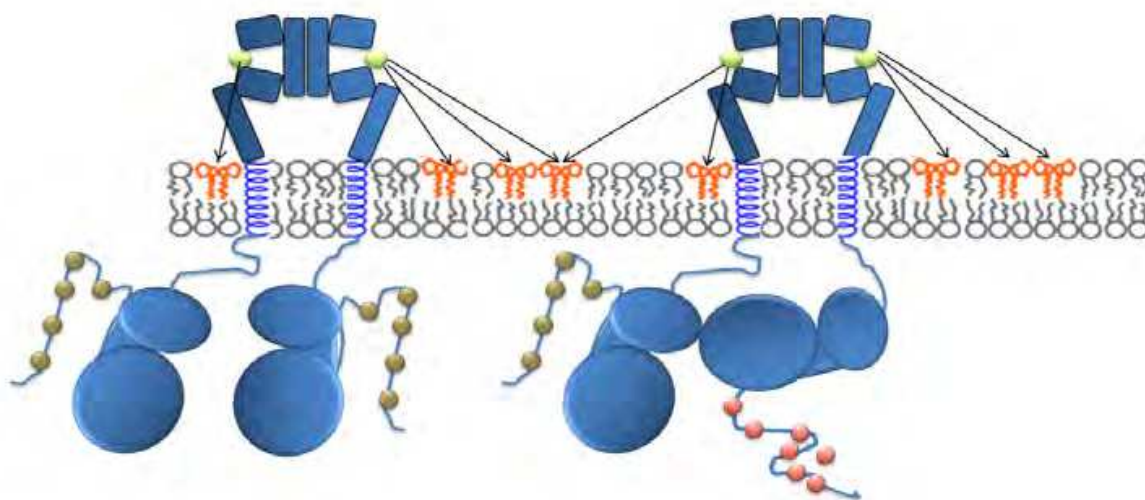
Fig. 5. A cartoon illustrating the FRET problem in two dimensions. Two receptor dimers are depicted bound to donor-labelled EGF (green spheres). The plasma membrane is labelled with lipid acceptors (red). Many distances are possible between each donor and the acceptors in the membrane. By varying the concentration of acceptors at the plasma membrane the vertical distance from donors to the cell surface can be calculated (Tynan et al. 2011).

The three examples cited were derived from cell-averaged steady-state intensity measurements (e.g., flow cytometry). The efficiency of FRET between a random distribution of donors and acceptors can also be determined using fluorescence lifetime imaging microscopy (FLIM). This method images with optical resolution the shortening of the fluorescence decay time of donor-labelled EGF induced by the lipid acceptors. Being a time-resolved assay, FLIM has the advantage of being able to directly measure the time between the absorption and emission of individual photons in the FRET donor fluorophores, providing results largely free from artefacts such as photobleaching and radiative transfer which can increase the errors in the measurement (Martin-Fernandez et al. 2002).

Using the combination of FLIM and FRET to measure protein-membrane distances, Webb et al obtained initial evidence for two types of EGF/HER1 complexes, tilted and upright with respect to the cell surface, that are associated to high-affinity and low-affinity EGF binding, respectively (Webb, et al. 2008). Subsequently, using MD simulations Kästner et al. showed that, with minor rearrangements, the HER1 back-to-back dimer can be aligned almost flat on the cell membrane, leading to conformational changes which further stabilize the extracellular dimer (Kästner et al. 2009). This work also showed that alignment on the cell surface and the interactions between the HER1 dimer and the upper leaflet of the membrane that follow break the pseudo-2-fold symmetry of the HER1 extracellular region, resulting in a highly asymmetric HER1 dimer structure.

12. HER1 can adopt key features of dEGFR asymmetry

To investigate the relevance of the MD-derived asymmetric model of the HER1 dimer relaxed on the membrane (Kastner et al. 2009) to EGF-binding heterogeneity and negative cooperativity (Macdonald & Pike 2008)), Tynan et al extended the FLIM-FRET assay

previously reported (Webb et al. 2008) to include FRET titrations as a function of acceptor concentration together with a data analysis method based on Monte-Carlo simulations (Tynan et al. 2011). This method allowed full quantification of the distance of closest approach between HER1-bound ligands and the surface of adherent epithelial cells, the assessment of the variation associated to the distance measurement, and also the rejection of potential sources of artifacts resulting from non-uniform distributions of FRET donors and acceptors.

Given the known flexibility regions in the accepted crystallographic HER1 ectodomain dimer structure, the FRET-derived distance data obtained from HER1 that display high-affinity for EGF (< 4 nm) could only be reconciled with HER1 structural data if receptors are aligned flat on the membrane (Fig. 6). MD simulations of doubly-liganded, singly-liganded and unliganded HER1 dimers aligned on a model membrane under the same conditions revealed that the asymmetry resulting from alignment on the membrane shares a number of key features with the equivalent doubly-liganded and singly-liganded structures observed recently in soluble dEGFR (Alvarado et al. 2010). These results suggest that the structural basis for negative cooperativity is conserved from invertebrates to humans but that in HER1 the extracellular region asymmetry requires interactions with the plasma membrane.
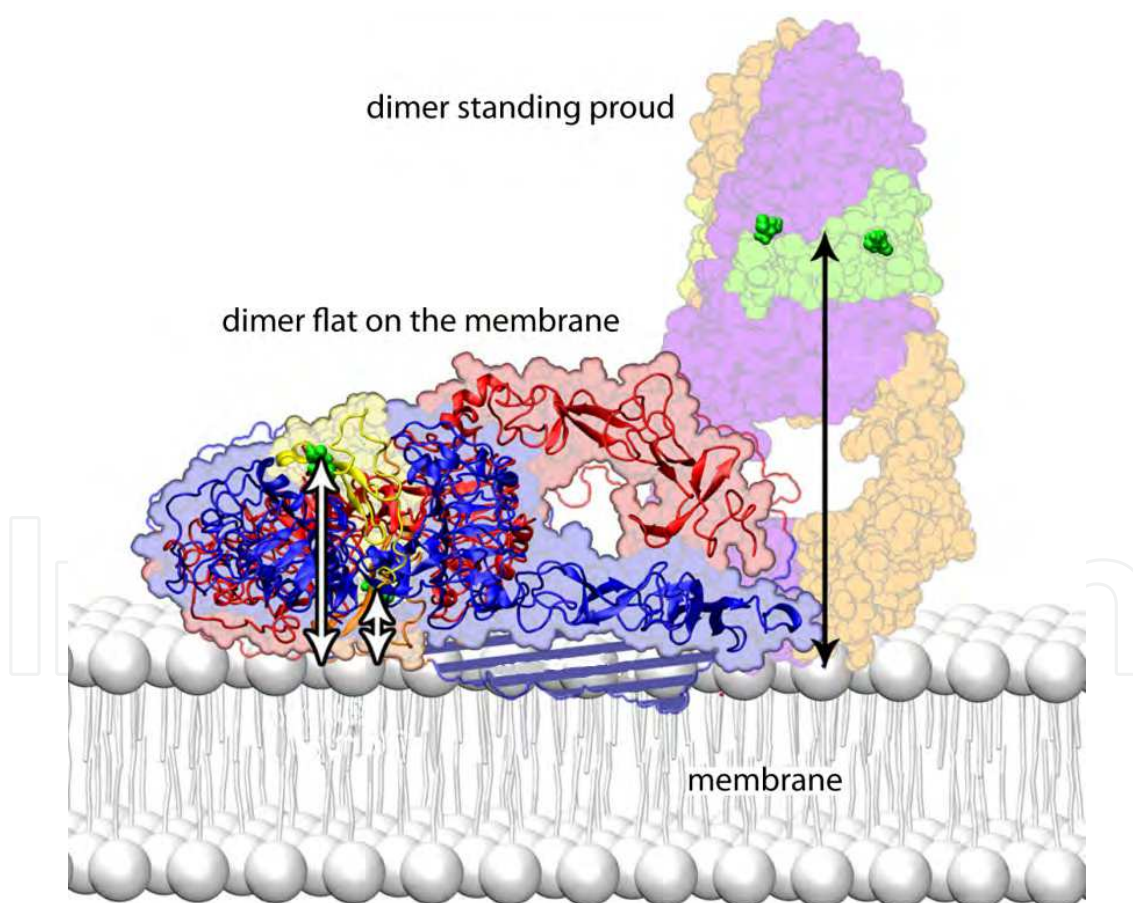

Fig. 6. The EGFR ectodomain dimer with two bound ligands, modelled on crystallographic structures (Garret et al. 2002; Ogiso et al. 2002; Ferguson et al. 2003) and placed in the membrane. Darker green spheres indicate the N termini of the ligands to which donor dyes are attached. Configurations standing upright and lying down are compared.

13. Imaging membrane protein interactions at the nanoscale

Understanding the structure-function relationships of biological macromolecules ultimately requires us to determine molecular structure at a range of resolutions. These include: Atomic resolutions (~1 Å) for detailed protein structure, derived from x-ray crystallography (Vrielink & Sampson 2003); 1-10 nm for measurement of conformations and inter-molecular distances, a range covered by electron microscopy (Agronskaia et al. 2008) and FRET (Stryer & Haugland 1967); and 10-20 nm for measurement of inter-unit separation and therefore the oligomerisation states of protein complexes. Given the importance of HER homo and hetero-oligomerisation in signalling, access to distances in the 10-20 nm range is crucial in investigation of HER interactions because, as discussed above, structural studies indicate that the inter-unit separation in the preferred receptor dimer should be in the region of 10-15 nm (Fig. 3) (Garrett et al. 2002; Ogiso et al. 2002). However, measurements in this range are challenging because these distances fall in a "resolution gap" between FRET and optical microscopy, which is diffraction-limited around 200 nm at best.

In recent years, a number of so-called "super-resolution" optical methods have been developed, that break the diffraction limit for light microscopy. These include stimulated emission depletion (STED) microscopy (Hell 2003) near-field scanning optical microscopy (NSOM) (Dunn 1999), photo-activated localization microscopy (PALM) (Betzig et al. 2006), fluorescence imaging with one-nanometre accuracy (FIONA) (Yildiz & Selvin 2005), single-molecule high resolution imaging with photobleaching (SHRImP) (Balci et al. 2005), nanometre-localized multiple single-molecule (NALMS) microscopy (Qu et al. 2004) and single-molecule high resolution co-location (SHREC) (Churchman et al. 2005). Theoretically, many of these techniques have the potential to measure distances in the required range. However, there are challenges in applying them to the cellular environment. NSOM is not well-suited to the wet conditions required for living or lightly fixed cells. STED and PALM are essentially ensemble imaging techniques that are not easily applied to the measurement of the distance between two or more specific molecules in the crowded cell environment. SHRImP, SHREC, and NALMS can measure distances between molecules with better than 10 nm resolution. Like PALM, all these methods "beat" the diffraction limit by imaging single molecules, fitting the point spread function (PSF) of the microscope, and locating its centre with nanometre accuracy. NALMS uses single molecule detection to count discrete steps in traces of fluorescence intensity vs time from diffraction limited spots, each step corresponding to the activation or bleaching of a single fluorophore, and measuring the change in the PSF of the spot before and after a step. In SHRImP, global fitting is carried out on spots before and after bleaching, producing nearly identical results to the sequential NALMS method. SHREC again uses PSF fitting, but two different fluorophores with different spectral characteristics are used, imaged in distinct channels which must be very accurately registered to determine intra-molecular distance. In all these techniques, spatial resolution is ultimately limited by the signal-to-noise ratio of the data, which determines the precision with which the centre of the PSF can be determined. For this reason, SHRImP, SHREC, and NALMS have so far been demonstrated only in "clean" samples such as purified, immobilised molecules on glass, and in the presence of antifade reagents.

The environment of mammalian cells is not conducive to the collection of high signal-to-noise data. The main source of background noise is intracellular autofluorescence, arising from molecules such as NADPH and flavins (Monici 2005). A common approach to reduce

background when looking at membrane proteins is to use total internal reflection (TIRF) excitation (Fig. 1) (Axelrod 1989). TIRF creates an evanescent field on the coverslip on which the cells are cultured. The field reduces exponentially with depth, and only penetrates approximately 300 nm into the sample. Thus, fluorescence is not excited in the bulk of the cell, reducing autofluorescence. This enables single molecule detection, as shown in Fig. 7 (left panel). Single molecules are clearly visible, but there is also a contribution from background noise. This results from residual autofluorescence, scattered light, and fluorescence from out-of-focus fluorophores.

In our laboratory we are developing new methods to improve the accuracy of intra-molecular distance measurement in cells and tissues. We currently achieve sub-10 nm positioning resolution in cells by using new data fitting algorithms that perform well in the noisy environment of cells (manuscript in preparation). Crucially, our techniques give robust estimates of errors, allowing the significance of distance measurements to be properly determined. Fig. 7 (right panel) shows typical single molecule traces and accompanying position measurement from labelled HER1 complexes in fixed epithelial cervical carcinoma HeLa cells.
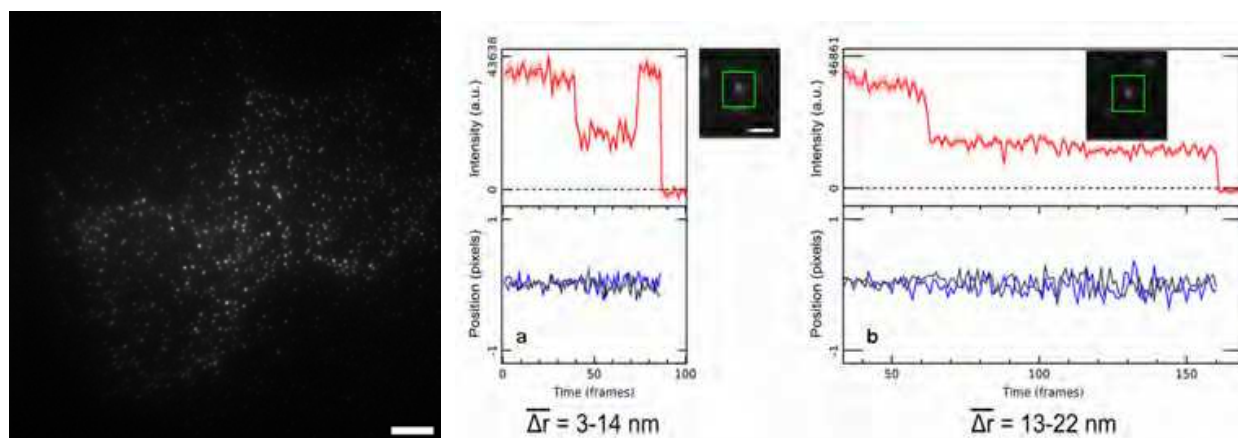

Fig. 7. *Left:* Single molecule TIRF image of EGFR in the plasma membrane of HeLa cells. EGFR are labelled with their ligand EGF, conjugated with the fluorophore Atto 647N (bar 8 μm). *Right:* Traces of single molecule fluorescence intensity (red) vs time for two example spots in fixed HeLa cells labelled with EGF-Atto 647N. a) shows a relatively short intra-molecular distance, b) a longer distance. Fluorophore x and y positions are plotted in black and blue, respectively.

14. Determining dynamic interactions from diffusion at the membrane

HER molecules interact with each other and with other network proteins while being embedded in the lipid bilayer of the plasma membrane, which is composed of neutral, charged, saturated and unsaturated lipids (Fig. 1). The lipids in the membrane consist of a hydrophilic charged region, the phospholipids heads, which form the extreme outer and inner leaflets of the membrane, and hydrophobic C-H chains of different lengths (typically from C_{12} to C_{20}) that forms the core of the bilayer (van Meer 2005). Water molecules concentrate on the inner and outer surfaces of the plasma membrane at the boundaries with the extracellular and intracellular regions and can occasionally enter the bilayer through

surface defects created by lipid mismatch. Hydrophilic protein regions are excluded from the hydrophobic inner part of the lipid bilayer and stick out from the plasma membrane.

The simplest membrane model consists of a 2-D liquid model surrounded by a 3-D solvent (Singer & Nicolson 1972). In this simple model, membrane proteins diffuse freely in the lipid bilayer which is modelled as a viscous liquid. Proteins interact with the surrounding lipid 2-D 'liquid' and diffuse with a speed that depends on lipid viscosity. However, it is observed experimentally that the lateral mobility of proteins in biological membranes can be orders of magnitude slower than in synthetic membranes. Reasons for this include:

i.   The presence of lipid rafts: The plasma membrane is composed of different lipids (e.g. phospholipids, sphingolipids and cholesterol) that can form microdomains of different viscosity. These rafts microdomains concentrate certain proteins (including HER molecules) and exclude others (see for example (Kusumi et al. 2005; Nagy et al. 2002).

ii. Intra-membrane barriers: Biological membranes are quite crowded with 15-35% of surface area occupied by many different types of proteins which can interact with each other affecting diffusion mobility (Scheuring & Sturgis 2005).

iii. Skeletal interactions: Proteins are able to interact with protein scaffolds and the cytoskeletal network beneath the plasma membrane in the intracellular side, which have the effect of corralling the membrane proteins in some regions and not others (reviewed in Costa et al. 2011).
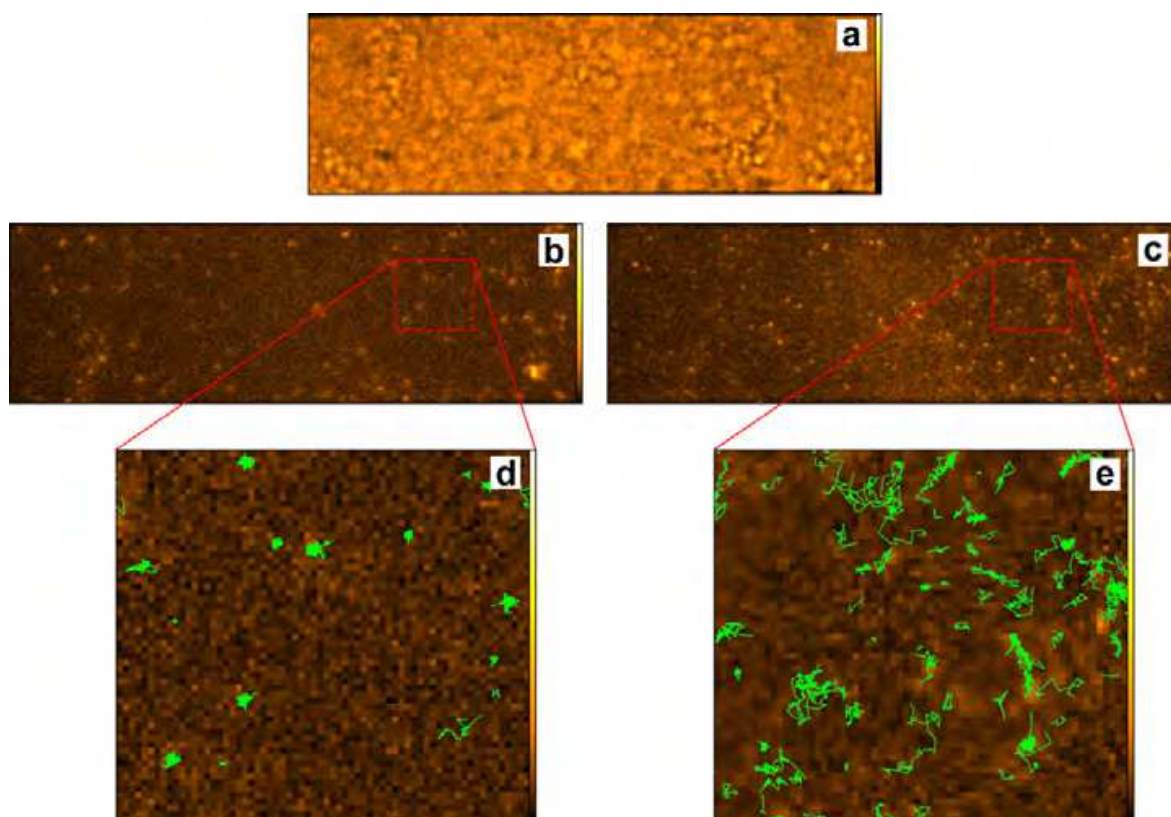

Fig. 8. Two-colour single molecule tracking of T47D cells. a) White light transmission image; b) cells labelled with 0.1 nM anti-ErbB2 affibody-Atto 647N; c) cells labelled with 2 nM anti-EGFR affibody-Alexa 488; d and e) single molecule tracks (green) from the spots located within the boxes marked in b and c.

The role of membrane microdomains, intra-membrane barriers and skeletal interactions in HER signalling is not yet understood. This role can be investigated using single molecule tracking. Advantages of a single molecule approach include the ability to observe dynamic, stochastic behaviour, such as compartmentalized diffusion (Andrews et al. 2008; Dahan et al. 2003; Fujiwara et al. 2002) that would be masked in ensemble measurements, and the ability for localisation of molecules with a precision well below the diffraction limit of light (see section 11).

To investigate protein diffusion and protein-membrane interaction in the HER bio-system we have used, for example, a two colour tracking system (Clarke et al. 2011) to follow the movements of HER1 and HER2 at the plasma membrane of live T47D cells, a human ductal breast epithelial tumour cell line cell model that expresses the four HER receptor types at a level between 10,000-30,000 receptors per cell. Fig. 8 shows single molecule images of HER1, and HER2 in living cells in their basal state. The receptors are labelled with an anti-HER1 affibody (Nordberg et al. 2010) conjugated with Alexa 488 an anti-HER2 affibody (Tran et al. 2007) conjugated with Atto 647N. The data were analysed using Bayesian segmentation algorithms and other data analysis methods described in (Rolfe et al. 2011). The resulting tracks indicate that HER1 displays in these cells higher mobility at the plasma membrane than HER2. We are now developing the methods to extract from these data the different mean displacements of each receptor in the plasma membrane from which their co-localisation kinetics can be derived.
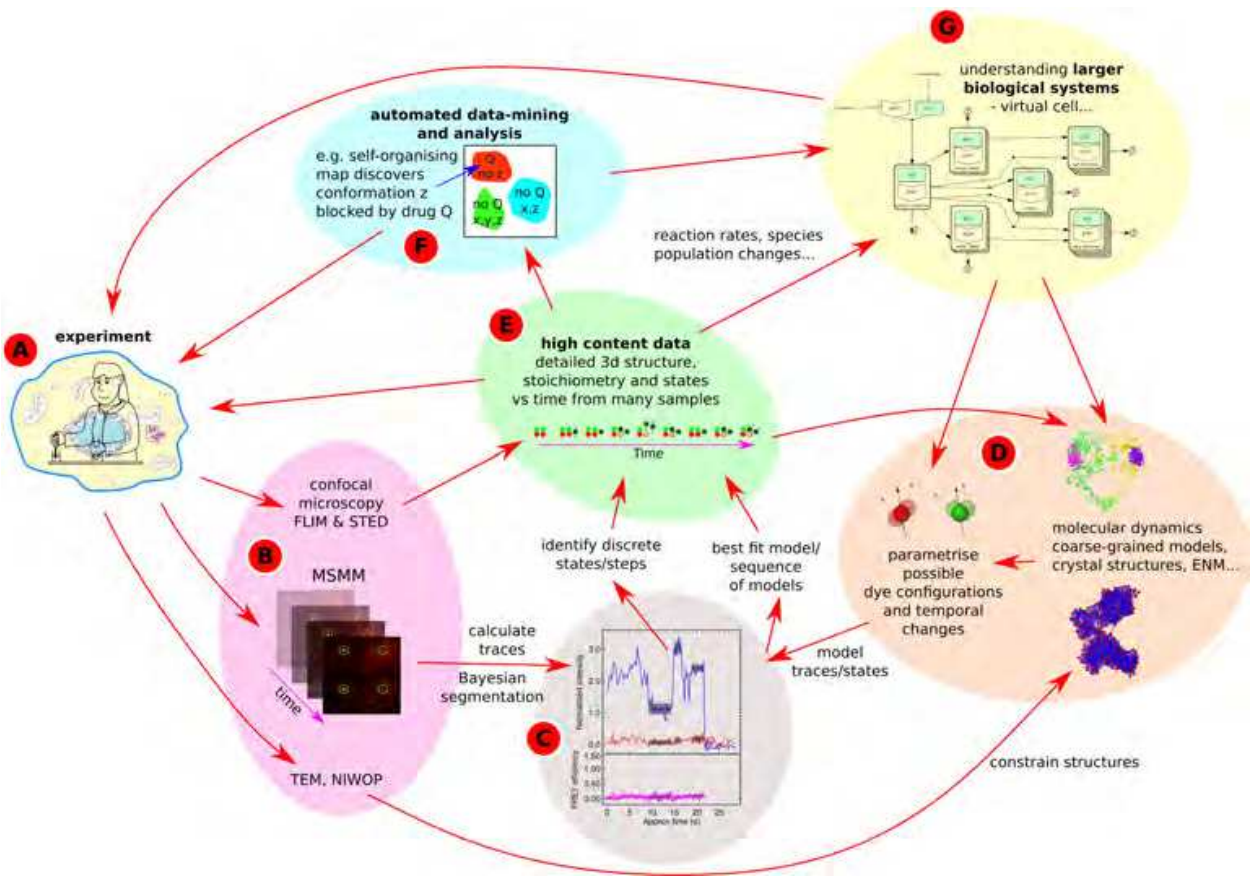

Fig. 9. Experimental and computational methods to investigate the HER signalling network

15. Towards an integrated approach

Ultimately, our goal is to use a cross-disciplinary portfolio of experimental and data analysis techniques to describe the HER signalling network not just in cell models but also in the real world setting. To achieve this we aim at deriving models of ligand-induced behaviour exploiting combined single molecule imaging, ensemble FLIM analysis and systems predictions. We also aim at establishing how HER signalling is influenced by feed-back loops and internal and external perturbations, e.g. HER mutations (seen in human subjects), RTK inhibitors and function-blocking antibodies. We also plan to combine experimental approaches with molecular dynamics and coarse-grained simulations and to explore data mining techniques for exploiting this rich data (Fig. 9).

16. View to the future: Potential clinical applications and take home message

We ultimately aim to refine single molecule fluorescence detection to be applicable to human tissues. We have already demonstrated single-molecule fluorescence imaging of HER receptors in fixed and frozen tissue sections from mouse tumour xenografts and human tissue biopsies (manuscript in preparation). This work is being done in collaboration with Prof. Peter Parker and colleagues at King's College London/Guy's & St Thomas' NHS Foundation Trust. Our aim is to extend the use of fluorescently labelled ligands, affibodies, anti-HER monoclonal antibodies (mAbs) and function blocking antibodies from cultured cell models to investigations of HER signalling on normal primary cells and tissue sections. We also plan to study naturally occurring somatic mutations (e.g. in human lung adenocarcinoma, see review on HER1 mutations in: Lynch et al. 2004) and also study cross-talk interactions with other non-HER receptors in lung and breast cancer tissue. It is hoped that these experiments will allow us to obtain further insights into the HER network and assess predictions made by our models. Once the methods have been optimised, studies will be extended to include other carcinoma cells and tissues, including matched normal tissue. Downstream targets of receptor activation will be assessed by western blotting to set the baseline population behaviour and ensure specificity of reagents. It is hoped that results from these experiments will provide novel insight on how the HER network operates at the tissue level and the supra-molecular rules and controls that are necessary to ensure normal network function.

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18. References

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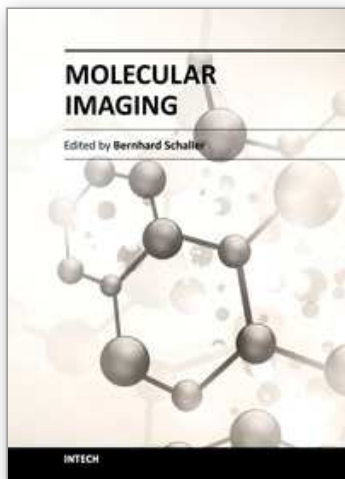
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Edited by Prof. Bernhard Schaller

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