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# Single-molecule fluorescence detection of the epidermal growth factor receptor (EGFR) in membrane discs

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#### Abstract

The epidermal growth factor receptor (EGFR) is critical to normal cellular signaling pathways. Moreover, it has been implicated in a range of pathologies, including cancer. As a result, it is the primary target of many anti-cancer drugs. One limitation to the design and development of these drugs has been the lack of molecular-level information about the interactions and conformational dynamics of EGFR. To overcome this limitation, this work reports the development and characterization of functional fluorescently-labeled full-length EGFR in model membrane nanolipoprotein particles (NLPs) for *in vitro* fluorescence studies. To demonstrate the utility of the system, we investigate ATP-EGFR interactions. We observe that ATP binds at the catalytic site providing a means to measure a range of distances between the catalytic site and the C-terminus. These ATP-based experiments suggest a range of conformations of the C-terminus that may be a function of the phosphorylation state for EGFR. This work is a proof-of-principle demonstration of single-molecule studies as a non-crystallographic assay for EGFR interactions means that new tools at the molecular level have the potential to significantly enhance our understanding of receptor pathology and are of utmost importance for cancer-related drug discovery.

### **Graphical abstract**

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#### Introduction

Receptor tyrosine kinases (RTKs) are critical for normal cellular signaling, development and homeostasis [1]. For example, regulation of cell proliferation, survival and differentiation is strongly mediated by the ErbB family of RTKs, of which the cell-surface epidermal growth factor receptor (EGFR) is a prominent member [2]. Overexpression and aberrant signaling of EGFR lead to pathologies, including various forms of cancer [3], which has motivated the development of a variety of inhibitors that serve as cancer therapeutics [4]. While initially effective, cancer cells are known to develop strong resistance to EGFR targeted therapeutics [5, 6].

Structural and biochemical studies have revealed that EGFR exists in both inactive and active forms [7–11] and have already mapped out the activation pathways. EGFR binds multiple extracellular ligands to form homodimers and three functional heterodimers, enabling the recruitment of intracellular signaling molecules [12]. EGFR binds adenosine-triphosphate (ATP) within the intracellular compartment at the catalytic site as both a monomer and dimer, suggesting that the monomer alone could also be active. Multiple sites for phosphorylation have also been reported [13], many of which are available in the monomer [14]. The molecular basis of ATP affinity has been extensively explored, including a series of mutations in the kinase domain to both increase and decrease ATP affinity [15, 16]. These results are particularly interesting because competitive inhibitors, such as Gefitinib, are widely used cancer therapeutics [17]

These studies of ATP binding have primarily used X-ray crystallography and have been limited to the truncated intracellular domains. However, deriving structural models from X-ray crystallography can lead to ambiguities associated with the identities and positions of receptor binding partners. Moreover, the observed conformations can be affected by the crystallization conditions, which may have important consequences for drug design. Finally, the dynamic nature of the interactions cannot be reported in real-time [18]. Consequently, much less is known about the transient conformational states that may exist during ligand-induced signaling events [19].

To address these three issues, single-molecule fluorescence techniques have emerged as powerful tools for non-invasive real-time probing of conformational transitions and molecular recognition events [20, 21]. EGFR has been modified to attach fluorescent dyes

for ensemble spectroscopy studies [14]. However, there have only been a handful of reports demonstrating single-molecule detection, the majority of which have been limited to live cell imaging. These studies have reported the presence of EGFR complexes on the surface of living cells [22] and identified the kinetics of ligand binding and the subsequent formation of dimers and multimers on the cell surface [23, 24, 25]. Additionally, ligand-binding and dimerization kinetics have been measured in the presence of inhibitors [26 – 29], and particle tracking experiments have revealed previously unknown intermediate states during EGFR endocytosis [30].

While insightful, these single-molecule experiments were performed in the living cell, which inherently limits elucidation of the molecular building blocks of EGFR signaling. For example, background extraneous processes that contribute to or mask the biochemistry of interest must be fully accounted for, cell autofluorescence in the visible spectrum can mask signals from the label, and long observation times are not easily achieved because of a marked reduction in dye photostability [31]. Conversely, experiments performed outside the living cell have traditionally introduced additional obstacles. Specifically, truncated domains may exhibit altered dynamics or functionality as well as lack the full signaling response. Furthermore, isolation of full-length receptors from live cells is problematic because solubilizers are known to compromise their conformation and stability [32, 33]. Reconstitution of EGFR into proteoliposomes has been achieved to systematically probe EGFR function [34]. However, despite the significant advantages that proteoliposomes provide over conventional methods, there are well-reported limitations with regards to use of their use [35]. Liposomes are often large, unstable, difficult to prepare with precisely controlled size and stoichiometry, and only offer access to one side of the membrane.

Consequently, cell-free expression techniques are rapidly evolving as an alternative option for protein production and are now often the primary choice for the synthesis of difficult targets [36], toxic proteins [37, 38], small bioactive peptides [39], membrane-bound receptors [40 - 42] and labeled proteins [43]. Here, the protein translation machinery of model organisms is used to express the protein of interest from an input cDNA sequence [44]. Cell-free expression has recently been used to produce soluble and functional ErbB receptors, including EGFR, reconstituted within 10-25 nm model-membrane nanolipoproteins (NLPs) [45]. The amphiphilic property of the NLPs provides a supported membrane mimetic that allows EGFR to be probed in a controllable, tunable and watersoluble environment, in the absence of any extraneous biochemical processes [46].

In this work, we overcome the obstacles posed by live cell imaging, sample heterogeneity and detergent-induced solubilization by building on the cell-free expression protocol [45] to produce fluorescently-labeled, full-length EGFR reconstituted in lipid NLPs. We demonstrate the biochemical production of the NLPs, full photophysical characterization and their single-molecule detection. Full-length monomeric EGFR supported by NLPs can be immobilized and fluorescence from EGFR monitored over extended time periods by confocal scanning microscopy or they can be detected freely diffusing in solution via multiparameter fluorescence detection and fluorescence correlation spectroscopy. To demonstrate the utility of our platform, we explore adenosine triphosphate (ATP) interactions with EGFR. We observe ATP binding and measure a range of distances from the C-terminus.

These results suggest heterogeneity in the conformation of the C-terminus, which may be due to phosphorylation state, as observed previously [14]. The ability to perform *in vitro* single-molecule experiments on the full-length receptor, introduced here, will enable non-invasive studies of transient and/or heterogeneous processes to facilitate the design of cancer therapeutics that target EGFR interactions.

#### Methods

#### Plasmids

ApoA1 49 was cloned into expression vector PD451-SR. The genes for EGFR were codon optimized for *E. coli* expression by DNA 2.0, and the SNAP gene was fused to the C-terminal of EGFR. A schematic of EGFR-SNAP, the *E. coli* codon optimization of human EGFR, and the cDNA and protein sequences of ApoA1 49 are shown in Figure S1. The fused EGFR-SNAP was cloned into expression vector pJexpress414 (DNA 2.0)

#### **DNA transformation and extraction**

1 μg EGFR-SNAP DNA was transformed into DH5-α cells (BioPioneer Inc.). EGFR-SNAP DNA extraction and purification was performed using the Plasmid Maxi Kit (Qiagen). ApoA1 49 DNA extraction and purification was performed using the Plasmid Mini Kit (Qiagen).

#### Cell Free Expression of EGFR-NLPs

Cell-free expression of EGFR-SNAP NLPs was performed using the Expressway<sup>TM</sup> Maxi Cell-Free E. Coli Expression system (Life Technologies). Codon-optimized cDNA plasmid encoding full-length EGFR tagged at the C-terminus with a SNAP-tag was subcloned into expression vector pJexpress-414 (DNA 2.0) and used for protein expression. The SNAP-tag is a 20 kDa mutant of the human DNA repair protein O6-alkylguanine-DNA alkyltransferase (hAGT) that reacts specifically and rapidly with benzylguanine functionalized derivatives, enabling the covalent labeling of the SNAP-tag with a synthetic probe [47]. ApoA1 49 in expression vector PD451-SR was used as the apolipoprotein belt. Since the SNAP tag does not compete with ligand binding and has no antagonistic function, the effects of ligands or inhibitors on EGFR can be studied [48]. E. coli slyD-extract, IVPS E.coli reaction buffer (minus amino acids), amino acids (minus methionine), methionine, T7 enzyme mix, DNA templates and small unilamellar 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids) vesicles were added to a 1.5 mL microcentrifuge tube to a final volume of 100µL and incubated at 30°C for 30 minutes at 500 rpm. A feed buffer made from IVPS feed buffer, 75 mM amino acids (-methionine) and 75 mM methionine was subsequently added to the reaction. The reaction was incubated for 18 hours at 30°C and stored on ice prior to purification. Small unilamellar vesicles were prepared by sonicating a 25 mg/mL water solution of DMPC phospholipids until optical clarity was achieved. The lipid concentration was calculated using a phosphorus assay [49] (Figure S2) and lipids were added to the cell-free reaction at a final concentration of 2 mg/mL. 1 µg of EGFR-SNAP plasmid DNA and 0.05  $\mu$ g ApoA1 49 DNA were added to the lysate. 2  $\times$  protease inhibitor (ThermoFisher Scientific) was also added at the beginning of the reaction. The reaction was

incubated at 30°C according to the manufacturer's instruction. The entire incubation time was 18 hours.

#### Native Purification of NLPs

200  $\mu$ L Ni-NTA resin slurry (Qiagen) was added to a 2 mL plastic column. Columns were thoroughly rinsed with ultra-pure water and equilibrated with 3 mL native lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) under gravity. Approximately 500  $\mu$ L of lysis buffer was left on top of the column. The completed cell free reaction was added to the prepared Ni-NTA resin and mixed overnight at 4 °C on a nutator. Flowthrough was collected and the column was washed with 2 × 1 mL native lysis buffer containing 10 mM imidazole, 2 × 1 mL native lysis buffer containing 25 mM imidazole and 2 × 1 mL native lysis buffer containing 50 mM imidazole. Samples were then eluted with 6 × 1 mL native lysis buffer (pH 7.5) using 10 kDa Slide-A-Lyzer Dialysis Casettes (ThermoFisher Scientific). Fresh buffer was added every hour in the first 3 hours. Samples were concentrated by centrifuging the sample in 30 kD columns at 4000 rcf for 10 minutes at 4 °C. Stock solutions were stored at 4°C.

#### SDS-PAGE

SDS-PAGE was performed using 12 % Mini-PROTEAN<sup>®</sup> TGX<sup>TM</sup> stain-free protein gels (10-well, 30  $\mu$ L) from Bio-Rad. Samples were boiled for 5 minutes with 2× Laemmli Sample Buffer (Bio-Rad) supplemented with 2.5 % 2-mercaptoethanol (Sigma Aldrich). Gels were run at 170 V for ca. 45 minutes. A pre-stained molecular weight marker was used to determine the end-point of the electrophoresis. Imaging was performed using Gel Doc<sup>TM</sup> imager (Bio-Rad).

#### Fluorescent labeling of EGFR-NLPs

50 nmol SNAP surface 594 (New England Biolabs) was dissolved in 50 mL anhydrous dimethyl sulfoxide (DMSO) (Sigma Aldrich) and mixed by vortexing for 10 minutes to yield a labeling stock solution of 1 mM SNAP-tag substrate. This stock was diluted 1:4 in fresh DMSO prior to labeling to yield a 250  $\mu$ M stock. EGFR-SNAP NLPs and SNAP surface 594 were added to 1 × PBS buffer (pH 7.5) and 1 mM DTT at a 1:2 molar ratio (V<sub>T</sub> = 50  $\mu$ L). The labeling reaction was incubated in darkness for 30 minutes at 37°C in a shaking incubator (150 rpm). Unreacted dye was subsequently separated from labeled species by spinning the solution at 14,000 rpm at 4°C in 30 kDa centrifugation filters. Labeled concentrations were estimated by absorption spectroscopy.

#### **Ensemble Optical Spectroscopy**

Absorption spectra of SNAP-Surface 594 labeled EGFR-NLPs and Alexa647-ATP in solution (1 × PBS buffer, pH 7.5) were measured on an Epoch (Biotek) spectrophotometer. Final SNAP Surface 594 and Alexa647 concentrations were determined using extinction coefficients of 120,000 M<sup>-1</sup>cm<sup>-1</sup> and 270,000 M<sup>-1</sup>cm<sup>-1</sup> at  $\lambda_{max}$ , respectively. Corrected fluorescence emission spectra were collected using a Cary Eclipse fluorescence spectrophotometer (Agilent) with excitation wavelengths as specified in the main text.

#### Single Molecule Spectroscopy

EGFR-NLP stock solutions were diluted to 1-10 pM in  $1 \times PBS$  buffer (pH 7.5) containing 50 nM protocatechuate-3,4-dioxygenase (PCD), 5 mM protocatechuic acid (PCA) and 2 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). For immobilization experiments, the diluted solution was incubated in a flowcell consisting of a 25 mm diameter hybridization chamber (Grace Biolabs) sealed to a Ni-NTA coated coverslip (Microsurfaces, Inc.). NLPs were attached to the surface by interactions between the His-tag on ApoA1 49 and the Ni-NTA. Single-molecule measurements were carried out on a home-built confocal microscope as previously described [50]. Briefly, a Ti:Sapphire laser (Vitara-S, Coherent;  $\lambda_c$ = 800 nm,  $\lambda$  = 70 nm, 20 fs pulse duration, 80 MHz repetition rate was focused into a nonlinear photonic crystal fiber (FemtoWhite 800, NKT Photonics) to generate a supercontinuum and then filtered to produce excitation at 550 nm or 640 nm. The excitation intensity was ca. 450 nJ/cm<sup>2</sup> on the sample plane. Excitation and fluorescence collection were performed by the same oil immersion objective lens (UPLSAPO100X0, Olympus, NA = 1.4). Fluorescence emission was passed through a dichroic filter (SP01-561RU, Laser2000, for 550 nm excitation, FF01-629/56-25, Semrock, for 640 nm excitation) before being passed through a series of bandpass filters (BLP01-647R, Laser2000, FF02-685/40-25, Semrock, FF02-675/67-25, Semrock, ET700/75m, Chroma). Donor and acceptor wavelength bands were detected on avalanche photodiodes (SPCM-AQRH-15, Excelitas). Photon arrival times were recorded by a time-correlated single-photon counting module (PicoHarp 300, Picoquant). The instrument response function for the apparatus was measured to be 0.35 ns (full-width at half-maximum). Time resolved fluorescence decays were obtained with  $\lambda_{ex} = 550$  nm at 71  $\mu$ W. Decay parameters were obtained from the maximum likelihood estimation fitting of the variation in fluorescence intensity, I(t), deconvoluted with the instrument response function, to a monoexponential decay of the form  $I(t)=I_0 + ae^{-t/\tau} 0$  where  $\tau$  is the lifetime.

#### Single Molecule Photobleaching Analysis

Data analysis was carried out using laboratory written routines developed in MATLAB R2013a. Intensity trajectories were recorded using binned photon-by-photon detection. Population histograms were constructed from 100 fluorescence intensity trajectories. Individual trajectories were analyzed using a change point algorithm, whereby a recursive generalized likelihood ratio test determines the location of a photobleaching step based on individual photon arrival times, and expectation-maximization clustering and the Bayesian information criterion is applied to identify the number of photobleaching steps [51].

#### Fluorescence Correlation Spectroscopy

FCS measurements were performed on the same confocal microscope. Samples were diluted to 1-10 pM and samples were allowed to freely diffuse within a 25 mm diameter hybridization chamber sealed to a glass coverslip coated with polyethylene glycol (PEG, MW = 5000). The average laser power ( $\lambda_{ex} = 550$  nm) was 60 µW. Fluorescence signals were autocorrelated and 3 million photons were typically collected for each measurement. All measurements are reported for a temperature of 21°C and 30 % humidity. Autocorrelation functions, G( $\tau$ ) were fitted to equation 1 [52].

$$G(\tau) = C + \left(\frac{1}{N}\right) \cdot \left(\frac{1}{1 + \frac{\tau}{V^2 \cdot |\tau_D|}}\right) \left(\frac{1}{\sqrt{1 + \frac{\tau}{V^2 \cdot |\tau_D|}}}\right) \cdot \left(1 - \left|t_f\right| + t_f \cdot e^{-\frac{\tau}{|t_s|}}\right) \quad \text{Eq. 1}$$

where C is a constant,  $\tau$  is the lag time, N is the number of molecules in the confocal volume,  $\tau_D$  is the translational diffusion time, V is a measure of the detection volume defined as  $Z_0/w_0$ , where  $Z_0$  and  $w_0$  are the distances at which the 3D Gaussian volume has decayed to 1/e in the axial and radial directions, respectively,  $\tau_f$  is the triplet fraction and  $\tau_s$  is the triplet lifetime. The counts per molecule (CPM) were subsequently calculated using Equation 2,

$$CPM = \frac{1}{N}(1 - \tau_f) \quad \text{Eq. 2}$$

where I is mean intensity. FCS datasets were also fitted using a 2-diffusing species model of the form

$$G(t) = (1 - Y)G_1(t) + YG_2(t)$$
 Eq. 3

where Y represents the fraction of molecules associated with species 2. ATP solutions were prepared in  $1 \times PBS$  buffer, 1 mM MnCl<sub>2</sub>, 2 mM DTT, pH 7.5 and incubated with EGFR for 30 minutes prior to measurement. Human phosphor-EGFR antibodies capable of detecting EGFR phosphorylated at Y1068 were purchased from R&D Systems, USA, and used without additional purification.

#### Single Molecule FRET Analysis

Data analysis was carried out using laboratory written routines developed in MATLAB R2013a. Donor (SS594) and acceptor (Alexa 647) intensity bursts ( $I_D$  and  $I_A$ , respectively) were recorded using binned photon-by-photon detection. Energy transfer efficiencies, E, were calculated via

$$E = \frac{I_A - \beta I_D - \alpha I_A}{(I_A - \beta I_D - \alpha I_A) + \gamma I_D} \quad \text{Eq. 4}$$

where  $\beta I_D$  corrects for leakage of donor emission into the acceptor channel,  $\alpha I_A$  corrects for direct excitation of the acceptor, and  $\gamma$  accounts for differences between donor and acceptor detection efficiencies ( $\mu$ ) and quantum yields ( $\varphi$ ) [53]:

$$\gamma = \left(\frac{\mu_A}{\mu_D}\right) x \left(\frac{\varphi_A}{\varphi_D}\right) \quad \text{Eq. 5}$$

To ensure that FRET histograms contained monomeric EGFR-NLPs, a threshold algorithm was used to select molecules which displayed intensity bursts in the range 10-60 counts per 100 ms, a typical intensity distribution reflecting the intensity profile at the confocal spot.

#### **Transmission Electron Microscopy**

Cell-free expressed EGFR-NLPs were analyzed by negative stain transmission electron microscopy. 5  $\mu$ L droplets of sample in 1 × PBS buffer (pH 7.5) were added to glow-discharged carbon-coated 400 mesh copper grids and incubated for 5 minutes at room temperature to allow non-specific binding of the NLPs to the grids. The solutions were removed by gently blotting the side of the grid with filter paper. The grids were subsequently washed with 1.5 % aqueous uranyl acetate for 30 seconds, dried and analyzed in the electron microscope. NLPs were imaged with a Tecnai FEI transmission electron microscope (120 kV, 0.35 nm point resolution). Micrographs were selected to represent the average distribution, density and size of the NLPs. Disc-like objects were sampled using the forbidden line unbiased counting rule applied to quadrats positioned systematic uniform random on micrographs which were displayed in Image J [54]. The mean caliper diameter was measured both horizontally and vertically and the average of both measurements calculated for each structure.

#### **Results and Discussion**

#### Construction and Characterization of labeled EGFR NLPs

Plasmid encoding a  $6\times$  His-tagged version of human apolipoprotein A-I lacking the aminoterminal 49 (ApoA1 49) in expression vector PD451-SR was used to drive the spontaneous assembly of NLPs in the presence of 1,2-ditertradecanoyl-sn-glycero-3-phosphocholine (DMPC) vesicles as schematically shown in Figure 1a. The cell-free expression system produced full-length EGFR-SNAP and ApoA1 49 proteins in the presence of DMPC, after Ni-affinity purification, as detected by stain-free SDS-PAGE (150 kDa and 20 kDa, respectively) (Figure 1b). The production of NLPs was confirmed by transmission electron microscopy (TEM) and a quantitative analysis of the micrographs (Figure 1c,d) revealed an average diameter of 44.7 nm (N = 267) (Figure 1c). The hydrodynamic diameter of the NLPs in solution was further confirmed to be 30.1 nm by dynamic light scattering (DLS) (Figure 1e).

Prior to single-molecule evaluation, EGFR-SNAP NLPs fluorescently labelled with SNAP Surface 594 (SS594) were characterized in solution using ensemble steady-state optical spectroscopy, along with the free dye for comparison. The absorption spectrum of the labelled EGFR-SNAP NLPs is very similar to that of the free dye, with only minor changes in peak positions and profile (Figure 1f). Similarly, the fluorescence emission spectrum

(Figure 1g) was essentially identical to that of the free dye, indicating minimal photophysical perturbation of SS594 upon attachment to the SNAP-tag.

Single-molecule measurements were first made on the labelled EGFR-SNAP NLPs freely diffusing in aqueous solution by using confocal microscopy. Fluorescence correlation spectroscopy (FCS) of the NLPs at 1 nM in solution produced a correlation curve that could be fitted using the same model [49] as for the free dye, incorporating fluctuations caused by diffusion and triplet formation (Figure 2a). The recovered diffusion time for EGFR-SNAP NLPs is  $167 \pm 2 \,\mu$ s, whereas the value measured for the free SNAP surface 594 dye is 59.8  $\pm 1 \,\mu$ s. The fraction of molecules in the triplet state under both conditions was comparable (0.314 in the case of the free dye and 0.342 in the case of NLPs) and the recovered triplet state lifetime was 6.9  $\mu$ s and 2.5  $\mu$ s respectively (Table S1). The similarity in photon count rate per molecule for NLPs ( $82 \pm 3 \,\text{kHz} \,\text{mol}^{-1}$ ) and free dye ( $85 \pm 3 \,\text{kHz} \,\text{mol}^{-1}$ ) further demonstrates minimal photophysical perturbation of the dye post-attachment.

Time correlated single photon counting was performed in parallel to demonstrate that the photophysics of the SS594 was unperturbed post-labelling. Here pulsed laser excitation and simultaneous photon-counting allowed measurement of fluorescence intensity and lifetime from single-molecule photon streams as EGFR NLPs traversed the confocal volume. An ensemble analysis of this population (Figure 2b) reveals that the fluorescence decay can be fitted to a single exponential with a lifetime of  $1.85 \pm 0.09$  ns, indicating a single dye environment and the absence of dynamics on the millisecond timescale. This was comparable to the lifetime extracted from the free dye in solution ( $1.99 \pm 0.21$  ns) (Figure 2b). The presence of a single lifetime population indicates that there is a single labelled species that is unquenched. Collectively, this photophysical characterization demonstrates the utility of the construct for fluorescence studies.

Next, we demonstrate that the majority of NLPs contain monomers via stepwise photobleaching of SS594. NLPs were immobilized onto a Ni-NTA coated glass coverslip via the  $6 \times$  His tag on ApoA1 49 as showing in Figure 3a. This approach has been widely adopted for immobilizing NLPs to surfaces, avoiding the need for direct covalent attachment of the receptor and allowing long-term observation of the receptors within a near-native environment [55, 56]. The surface was imaged by raster scanning the sample across the confocal volume, allowing intensity and lifetime of single molecules to be collected sequentially. The number of molecules inside a particular disc was measured by recording the number of photobleaching steps in time traces of the fluorescence signal from individual diffraction-limited spots. This revealed levels of constant intensity followed by stepwise photobleaching of the fluorophores (Figure 3b). The stability of the signals before a bleaching event is notable, with no dynamics on the time scales studied, providing further evidence that the fluorophore is not perturbed by attachment to the SNAP tag, because any interactions will lead to quenched states in which the dye fluorescence turns off. This observation therefore allows a reliable estimate of the number of labelled EGFR receptors within a NLP. A total of 80 % of the traces displayed one-step photobleaching events while 16 % displayed two steps (Figure 3c). Fewer three- and four-step bleaching events were observed. Thus most of the detected NLPs contained only one EGFR receptor. An evaluation of the fluorescence intensity and on-times showed that at an excitation power of 180 nW, an

average of 50 counts/100 ms was obtained, and that the fluorophores photobleached over timescales of ca. 10 seconds (Figure S3). To demonstrate that the binding of the NLPs to the surface through the His/Ni-NTA interaction is long-lived, the excitation power was reduced 5-fold to 36 nW, resulting in prolonged intensity-time trajectories (Figure S4), indicating that the loss of fluorescence shown in Figure 3b is due to photobleaching and not dissociation from the surface. Moreover the lifetime distribution obtained from immobilized NLPs (Figure 3d) closely matched the ensemble value observed in solution, demonstrating negligible photophysical perturbation of single SS594 dyes post-attachment. It is possible that various EGFR conformations exist during the experiments, but these do not lead to substantial changes in lifetime, as may be expected during protein-induced fluorescence enhancement or quenching processes. This will enable researchers to accurately monitor changes in lifetime as a function of condition (e.g. via FRET) with confidence that photophysical changes are not caused by dye-protein interactions in the steady-state.

To assess the functional integrity of NLP bound SS594-labeled EGFR, we examined receptor phosphorylation by FCS using an antibody that specifically recognizes EGFR phosphorylated at tyrosine residue 1068. FCS is a well-established tool for identifying receptor-ligand binding [57], and because the *in vitro* translation reactions contain both the ATP and metal ion cofactors necessary for EGFR kinase activity, changes in the FCS diffusion time in the presence of antibody are a strong indicator of EGFR activity. After Niaffinity purification was performed to remove cell-free expression lysate components, the purified EGFR-NLPs were incubated with ATP, Mn<sup>2+</sup> and Y1068 antibody to allow for antibody-binding. FCS demonstrated that the diffusion time of 0.5 nM EGFR NLPs in phosphorylation buffer increased by  $20.4 \pm 1.1$  % in the presence of 1  $\mu$ M antibody, indicating that the purified EGFR NLPs are capable of phosphorylation (Figure 4a). In a complementary approach, the diffusion time of 250 pM Alexa647-labeled Y1068 antibody in the absence and presence of EGFR-NLPs was investigated. Here, the diffusion time of the labelled antibody (621 µs) was ca. 6-fold longer than that of the free dye (101 µs), and increased further to 643 µs in the presence of phosphorylated EGFR NLPs even when an antibody: EGFR molar ratio of 1: 4 was used (Figure 4b, Table S2). Importantly, the diffusion time of the labelled antibody remained invariant when incubated with empty NLPs (Figure S5). Evidence confirming the presence of resonance energy transfer between SS594 on EGFR and Alexa-647 on the Y1068 antibody were obtained in phosphorylation buffer from the decrease in the average fluorescence lifetime of the donor in the presence of the acceptor, which reduced by 4.9 %, 8.3 % and 36.4 % when EGFR: Y1068 antibody molar ratios of 2:1, 1: 2 and 1: 20 were tested, respectively (Figure 4c). The increase in FRET efficiency indicates that antibody binding occurs within the intracellular domain and is consistent with previous work that demonstrates EGFR functionality via ligand interaction when contained within supported membrane mimetics composed of DMPC phospholipids [45, 58]. The combination of FCS and FRET measurements are consistent with antibody binding to the intracellular compartment of functional EGFR and thus emphasize the potential of EGFR NLPs as a versatile platform.

#### Exploration of EGFR-ATP Interactions using FCS and Single-Molecule FRET

The production of single, fluorescently labeled EGFR monomers within NLPs enables studies of transient states and interactions, which we demonstrate by investigating the interaction between EGFR and ATP. We use two complementary fluorescence approaches: FCS and multi-parameter FRET detection. Both techniques confirmed binding ATP-EGFR interactions. The FCS diffusion times of freely-diffusing EGFR in NLPs in the absence and presence of 100 nM and 1  $\mu$ M ATP were found to be 167, 169 and 172  $\mu$ s, respectively (Figure 5a). These diffusion times correspond to a detectable increase in the average hydrodynamic radius, from 6.14 nm in the absence of ATP, to 6.21 nm and 6.32 nm in the presence of 100 nM and 1 µM ATP, respectively, and are attributed to ATP binding. No difference in the diffusion times without the standard error of the mean were observed when the ATP concentration was increased beyond the reported Km[ATP] [59] and Kd[ATP] [14] values for EGFR to 10 µM and 1 mM, respectively, indicating relatively high fractional occupancy was achieved at 1 µM (Figure S6). Fitting parameters associated with the application of Equation 1 to the FCS curves are shown in Table S1. Negligible dimeric/ multimeric EGFR NLPs were recovered when the FCS curves were fitted to a 2-diffusing species model indicated in Equation 3 (Y = 0.002) (Figure S7).

Evaluation of less concentrated samples of EGFR NLPs (1-10 pM) yielded intensity time traces, which exhibited clear single-molecule intensity bursts, providing a unique opportunity to evaluate EGFR-ATP interactions via FRET. As shown in Figure 5b, the fluorescence time trajectory obtained for 10 pM SS594 labeled EGFR NLPs (donor) in the presence of Alexa Fluor 647 (acceptor) labeled ATP shows coincident bursts of fluorescence. Over the duration of the measurements there were no observations of SS5994 bursts >60 counts per 100 ms, indicating that the FRET data collected refers to the interaction between ATP and the EGFR monomer only. Under direct excitation of SS594  $(\lambda_{ex} = 550 \text{ nm})$ , the appearance of fluorescence at both donor and acceptor wavelengths at the same time in the focal volume demonstrates the interaction of EGFR and ATP. For each fluorescence photon burst, a FRET efficiency, EFRET, was computed and collected into a histogram [60]. The histogram is centered on a FRET efficiency of  $0.44 \pm 0.02$  (FWHM =  $0.29 \pm 0.05$ ) corresponding to an estimated labeling site to phosphorylation site distance of  $8.61 \pm 0.39$  nm (Figure 5c). The estimated distance is in good agreement with the distance between the catalytic cite and C-terminus previously reported [14], when the labeling positions are taken into consideration.

The wide distribution in our FRET histogram suggests a range of conformations of the Cterminus. Previous work demonstrated a conformational change in the C terminus of EGFR based on the phosphorylation state, which may contribute to the width of the distribution observed here [14]. However, here we observe a range of FRET distances, indicating a broad range of conformations. These different conformations of the phosphorylated EGFR may play a role in modulating the nucleotide-binding properties of the kinase domain [61].

While in vitro analysis of monomeric EGFR provides a means to overcome complexity, recent evidence points to ligand-induced multimerization of EGFR [62], the assembly of which optimally organizes kinase-active dimers for auto-phosphorylation [63]. In this regard, the utility of the cell-free expression platform presented here may be extended to

enable active dimers and multimers of EGFR to be embedded within single NLPs [58] of defined diameter and radius of curvature [64, 65]. Relatively low expression yields (< 0.5 ng/ $\mu$ L) do mean conventional activity assays such as Western Blotting are ineffective, but nevertheless, the application of single-molecule techniques are rapidly evolving to enable such systems to be spectroscopically accessed.

#### Conclusions

We have reported the cell-free expression and detection of functional fluorescently-labelled EGFR-SNAP in model membrane NLPs, freely diffusing and immobilized, using singlemolecule spectroscopy. The fluorescent properties of the SNAP-substrate label were maintained upon conjugation to EGFR-SNAP, indicating the utility of this approach for studies of labeled receptors. The coupling of FCS and multi-parameter FRET detection with the development of labeled EGFR NLPs provides a facile yet powerful approach for quantitatively measuring interactions involving this important class of biomolecule in solution. Here, we simultaneously detect the presence of free and ATP-bound EGFR via changes in diffusion time and FRET. We observe a range of conformations of the C terminus, possibly due to the phosphorylation state. This work forms the basis of a new method for the investigation of the behavior of the EGFR-family of membrane receptors at the single-molecule level, which provides information that is currently hidden from ensemble averaging methods (such as NMR and X-ray crystallography) and live cell imaging, where extraneous background signals can hide the biochemistry of interest. This is a proof-of-principle demonstration of the single-molecule detection of EGFR within controllable, tunable and near physiological lipid NLPs and opens a platform for the screening of monoclonal antibodies and small molecule inhibitors associated with this important class of biomolecule.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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#### Figure 1. Characterization of fluorescently-labeled EGFR-SNAP NLPs

(a) Schematic representation of fluorescently labelled EGFR NLPs. (b) Image of denaturing SDS-PAGE gel of cell-free expressed ApoA1 49 and EGFR-SNAP post Ni-affinity purification and dialysis. (c) TEM micrographs of EGFR-SNAP NLPs negatively stained with 1.5 % (w/V) uranyl acetate. Scale bars = 50 nm (d) Size distribution histogram obtained from TEM micrographs. (e) Dynamic light scattering distribution obtained from EGFR-SNAP NLPs in 1× PBS buffer (pH 7.5). (f) Absorption spectra and (g) normalized fluorescence emission spectra of SNAP surface 594 labelled EGFR-SNAP NLPs (red) and free SNAP surface 594 (black) in solution (pH 7.5).



#### Figure 2. Characterization of EGFR-SNAP NLPs using FCS and TCSPC

(a) Fluorescence correlation curves of SS594 labelled EGFR-SNAP NLPs (red) and free SS594 in solution (black) (pH 7.5). (b) Time resolved fluorescence decays of the labelled NLPs (red) and free SS594 (black). Bold lines are single exponential decay fits. The grey line represents the instrument response function.

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**Figure 3. Single-molecule fluorescence detection of immobilized EGFR-SNAP NLPs** (a) Schematic of the immobilization scheme for attaching NLPs to a Ni-NTA surface via the his-tag on ApoA1 49. (b) Representative 1- and 2-step (blue) photobleaching trajectories. The black solid lines are fits to the data as determined by a changepoint algorithm. Histograms of (c) photobleaching steps and (d) lifetimes observed from immobilized NLPs in 1 × PBS buffer (pH 7.5) are also shown.





(a) FCS autocorrelation curves of 0.5 nM freely-diffusing EGFR NLPs in the absence (black) and presence (red) of 1  $\mu$ M Y1068 antibody in phosphorylation buffer ( $\lambda_{ex} = 550$  nm). (b) FCS autocorrelation curves of Alexa 647 free dye (green), Alexa647 labeled 1068 antibody in phosphorylation buffer in the absence (blue) and presence (red) of EGFR-NLPs ( $\lambda_{ex} = 640$  nm). (c) Time-resolved fluorescence decays of SS594 EGFR NLPs in the absence and presence of 1 nM (green) and 10 nM (blue) Alexa647-labeled Y1068 antibody ( $\lambda_{ex} = 550$  nm). Solid black lines represent exponential fits. The grey line represents the instrument response function.



Figure 5. Fluorescence correlation spectroscopy and FRET detection of EGFR NLPs in the presence of ATP  $\,$ 

(a) FCS autocorrelation curves of 1 nM freely-diffusing EGFR NLPs in the absence and presence of ATP at increasing concentrations. (b) Representative SS594 (donor, green) and Alexa647 (acceptor, red) intensity trace demonstrating co-localized single molecule bursts and (c) the corresponding FRET histogram.