OCTOPUS: A multi-laser facility for combined single molecule and ensemble microscopy


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Abstract

OCTOPUS (Optics Clustered to OutPut Unique Solutions) is a microscopy platform that combines single molecule and ensemble imaging methodologies. A novel aspect of OCTOPUS is its laser excitation system, which consists of a central core of interlocked continuous wave (CW) and pulsed laser sources, launched into optical fibres and linked via laser combiners. Fibres are plugged into wall-mounted patch panels that reach microscopy end-stations in adjacent rooms. This allows multiple tailor-made combinations of laser colours and time characteristics to be shared by different end-stations minimising the need for laser duplications. This set-up brings significant benefits in terms of cost-effectiveness, ease of operation, and user safety. The modular nature of OCTOPUS also facilitates the addition of new techniques as required, allowing the use of existing lasers in new microscopes while retaining the ability to run the established parts of the facility. To date, techniques interlinked are multi-photon/multi-colour confocal fluorescence lifetime imaging (FLIM) for several modalities of fluorescence resonance energy transfer (FRET) and time-resolved anisotropy, total internal reflection fluorescence (TIRF) single molecule imaging of single pair FRET (spFRET), single molecule fluorescence polarisation (smFP), particle tracking, and optical tweezers. Here we use a well studied system, the epidermal growth factor receptor (EGFR) network, to illustrate how OCTOPUS can aid in the investigation of complex biological phenomena.

I. INTRODUCTION

There is an ever-increasing range of microscopy techniques that use lasers as light sources. This plethora of methods reflects the multiple requirements of life sciences research, including nanometre spatial resolution, identification of molecular interactions, location of specific molecular species, in situ measurement of molecular dynamics and kinetics, and real-time tracking of cellular processes. However, despite the range of techniques now available, no individual method is able to fully reveal the multidimensional array of molecular information that is required to understand complex biological processes. For this reason, it is not unusual to find collections of laser-based microscopy systems of different types located in the same laboratory. Ideally, each of these
microscopes would be fitted with a range of laser sources providing maximum flexibility of operation with multiple fluorescent probes, and with multiple techniques requiring laser illumination with different characteristics, such as time structure, power, and polarisation. However, cost and space considerations generally restrict the number of laser sources that are dedicated to an individual microscope. This in turn restricts the range of possible experiments, for example limiting the choice of fluorescent probe, or not allowing the examination of the same sample using different techniques.

The OCTOPUS cluster is designed to optimise the combination of multi-technique ensemble and single molecule approaches to investigations of cellular processes. A novel design feature is the addition of a central laser hub. This is equipped with a cluster of laser sources that can be split and grouped via wavelength multiplexers to produce multi-wavelength outputs that can be simultaneously distributed to several microscopes in laboratories surrounding the laser hub. Multi-wavelength laser light combinations are delivered via fibre optics plugged to connectors in patch panels on common walls between laboratories. Using this system fibres are connected to microscopes in a ‘plug-and-play’ fashion to achieve the optimal laser excitation characteristics required by different experiments. This allows significant flexibility in the development and combination of imaging methodologies while reducing the need for duplicating expensive laser sources and minimising setting-up time.

The ensemble techniques currently available in OCTOPUS are confocal imaging, FLIM, FRET and time-resolved fluorescence anisotropy. Confocal and FLIM are used to investigate protein localisation and transport, and, environmental variations such as polarity, pH, and ion concentration. In combination with FLIM, FRET can detect and locate molecular interactions (FRET is extremely sensitive to intermolecular distances in the range 1-10 nm) while in combination with time-resolved fluorescence anisotropy, it can image local variations in the speed of molecular rotations. A Total Internal Reflection (TIRF) microscope equipped with optical tweezers allows simultaneous high-resolution imaging and manipulation of microscopic objects. The single molecule imaging methods in OCTOPUS generate complementary information to their ensemble counterparts. For example, single pair FRET (spFRET) can describe the distribution of distances around the mean reported by FLIM-FRET. SpFRET can also be combined with single molecule polarisation (smFP) to measure distances and angles between subdomains in a molecular complex, complementing time-resolved anisotropy measurements. Single molecule methods can also track individual molecular movement, complementing the information on molecular localisation and trafficking data derived from ensemble imaging.

Here we show the advantages of OCTOPUS in combined single molecule and ensemble investigations of the EGFR signalling network, illustrating how technique combination can provide complementary information on protein structure, conformational changes, protein-protein interactions and protein dynamics, kinetics, and trafficking.

II. MATERIALS AND METHODS

A. Cell culture. A431 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), without phenol red and supplemented with 10% Fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin. HeLa cells were cultured in Minimum Essential Medium (MEM) with Earle's salts, without phenol red, supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin (all Invitrogen). HEK-293 cells were cultured
in MEM with Earle’s salts, without phenol red, supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 µg streptomycin. T47D cells were cultured in RPMI 1640, without phenol red and supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine (all Invitrogen). Cell samples were deprived of serum for 16 hours upon reaching ~80% confluency. Cells were incubated at 37 °C in the presence of 5% CO₂ in air.

**B. Fluorescent ligands.** Alexa 488 and Alexa 546 (Invitrogen) and Atto 647N (Atto Tec) were conjugated to the N-terminus of murine epidermal growth factor (EGF) in a 1:1 stoichiometry (dye conjugation by Cambridge Research Biochemicals). EGFR affibody and ErbB2 affibody (Abcam) were labelled at single cysteine residues in a 1:1 stoichiometry following the manufacturer’s instructions. HEK-293 and A431 cells were transiently transfected with EGFP-Rheb vector using Fugene HD transfection reagent (Roche Applied Sciences, UK). 24 hours after transfection live cells were labelled with 200 nM EGF-Alexa 546. Fab fragments of the monoclonal antibody 29.1 (Sigma Aldrich, Gillingham, UK) were prepared and conjugated to Alexa 488 as described previously.

**C. Cell preparation and labelling.** Cells were incubated at 4°C for 30-60 minutes, or for 15 minutes at 37 °C for tracking experiments, with the appropriate fluorescence derivatives in phosphate-buffered saline (PBS). If required, cells were then fixed by incubation with 3% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at 4°C then 15 minutes at room temperature. If required, the membranes of pre-chilled A431 cells were partially depolarised by incubation with 100 µM Ouabain diluted in PBS for 10 minutes at 4 °C. The partial depolarisation was maintained whilst the cells were incubated in EGF by diluting EGF into PBS supplemented with 100 µM Ouabain. The total incubation in Ouabain was 40 minutes at 4°C. If required, EGFRs were maintained in a dephosphorylated state by treating cells with PBS supplemented with 10 mM 2-Deoxyglucose, 10 mM Sodium Azide, and 0.1 % Bovine Serum Albumin (BSA), and incubating with gentle rocking at 37 °C for 20 minutes. To label EGFRs with mAb 2E9-Alexa 488, samples were incubated with a 1 nM concentration of antibody at 4 °C for 4 hours with gentle rocking. To label cells with membrane probe, samples were incubated in 10 µM DiI diluted in serum free media for 10 minutes at 37 °C.

**D. FLIM Data analysis.** Fluorescent intensity decays were best fitted to a single exponential decay model where acceptor was absent and a bi-exponential model when both donor and acceptor were present, and distance measurement was derived from the data using Monte Carlo simulations. Calibration of acceptor density was done as described previously.

**III. RESULTS AND DISCUSSION**

**A. Layout and Laser Distribution**

The OCTOPUS cluster consists of a central laser hub, which houses a cluster of CW and pulsed laser systems, surrounded by microscopy laboratories (Fig. 1). The lasers available in OCTOPUS, are listed in Table I. The laser hub also houses a two-photon confocal microscopy system (Fig. 1a). This is the only microscope in OCTOPUS not linked by fibre optics because of stringent constraints on the time structure of the laser pulse for two-photon excitation. Laser radiation is supplied to the individual microscopes via fibres plugged into patch panels set into the wall between laboratories (Fig. 2a). Both mating sleeves (Newport and Thorlabs) and fibre-
fibre couplers (Schäfter and Kirchoff) are integrated into the patch panels to connect the fibres on each side of the wall; while the former are much cheaper, the latter have higher and reproducible transmission. The system is completely flexible and allows light from any hub laser to be supplied to any microscopy station located in the surrounding laboratories.

Laser lines are fibre-launched and combined according to the needs of the experiment to supply illumination with different wavelengths, pulse repetition and length. This is achieved through a combination of polarisation maintaining single mode optical fibres, fibre combiners, and the outputs from the Ti:Sa and OPO sources. Higher order harmonic generation can be used with the latter to generate ultra violet laser radiation when required. Fibre optic wavelength division multiplexers (Oz Optics) are employed to combine two or three laser sources. If the experiment requires multiple excitation wavelengths (e.g. multi-colour molecular tracking), supercontinuum lasers coupled to an acousto optical tunable filter (AOTF) (Fianium) can also be used to supply pulsed illumination at a number of wavelengths (up to 8) through a single fibre. The fibres transporting light into the microscopes are interlocked through a central control room that monitors which lasers are powered. The outputs from all the lasers can be divided and shared as required between all microscopes.

The potential to deliver a wide range of laser wavelengths, powers, and pulse structures remotely to a number of laboratories requires a comprehensive interlock system for the protection of microscope operators and users. The OCTOPUS cluster uses the Argus/Cerberus interlock system developed in the Central Laser Facility\textsuperscript{24, 25}. The system monitors the status of the lasers, patch panels, microscope enclosures, and laboratory doors, and shuts off laser radiation in the event of an unsafe condition. Control of lasers and shutters is provided in each laboratory through one or more touch screen Magelis Human Machine Interface control panels (Fig. 2b), while an overview of the status of the cluster is provided through the Cerberus information display system (Fig. 2c).

B. Microscope techniques embedded in OCTOPUS

1. Three-Colour Single Molecule tracking

This microscope has been developed to excite fluorescence in three colours and detect this in three channels. This is used, for example, to investigate interactions between protein species labelled with three different fluorophores, such as those interacting within a signalling network. The microscope can also be used to measure spFRET between, for example, one FRET donor and two acceptors. Its location is shown in Fig. 1(b).

The optical layout of this microscope is shown in Fig. 3a. Three lasers, for example with wavelengths $\lambda = 491\text{nm}$ (100 mW, Cobolt Calypso), $561\text{ nm}$ (100 mW, Oxxius SLIM), $639\text{ nm}$ (30 mW, PTI IQIC30), are combined via a polarisation maintaining wavelength multiplexer (Oz Optics) in the OCTOPUS hub. The output fibre of the multiplexer, which is also polarisation maintaining, is plugged into an inverted microscope (Zeiss, Axiovert 200M), via the patch panel, through a polarisation dependent TIRF slider that allows both TIRF and epifluorescence illumination. TIRF illumination is necessary for single molecule measurements in cells to reduce the level of background fluorescence from the sample. The laser beam is reflected into the objective (Zeiss, $\alpha$-plan fluar, 100× magnification, nominal N.A. 1.45) by suitable triple band filter sets (Chroma and Semrock). Fluorescence from the sample is imaged onto the entrance aperture of an Optosplit III image splitter (Cairn Research). This divides the image into three spectrally distinct but spatially identical components, which are imaged side by side on an electron multiplication CCD (EMCCD) camera (Andor iXon). Fig. 4 shows an
example of the simultaneous single molecule imaging of three fluorophores using this microscope: EGF labelled with Atto 647N, Alexa 546, and Atto 488, bound to EGFR in the plasma membrane of HeLa cells. Wavelength selection is determined by interchangeable dichroic beamsplitters and filters in the Optosplit III. An incubator (Pecon XL S1) encloses the sample stage to maintain a constant temperature and protect from air currents. This is important for nanopositioning experiments, where temperature instabilities result in drifts in sample position at the nanometre level. Data acquisition is controlled by custom LabVIEW software (National Instruments). This allows control of parameters such as EMCCD gain, frame duration, acquisition time, etc. Images are saved in HDF5 format for subsequent processing using custom-designed software. A second multi-colour microscope is currently under development (Fig. 1e), and will offer similar capability to the three-colour system, but allow simultaneous tracking at up to five different wavelengths.

2. **Eight-channel single molecule combined spFRET and smFP**

This microscope can be used with combined excitation laser light from the following CW laser pairs: \( \lambda = 491 \text{ nm} \) and 532 nm; \( \lambda = 491 \text{ nm} \) and 639 nm; \( \lambda = 532 \text{ nm} \) and 639 nm; \( \lambda = 561 \text{ nm} \) and 639 nm, and with combinations of the above CW lasers and the fibre-launched output from the OPO. It is located in the same room as the three colour tracking system described above (Fig. 1b) and shares the same patch panel connecting the room with the central laser hub. Pairs of lasers are coupled via a second wavelength multiplexer located in the OCTOPUS hub that is connected through the wall to the microscope (Zeiss, Axiovert 100) as described above. The optical path combining spFRET and smFP is as that previously described\(^{26}\). Briefly, the excitation light passes through a Glan-Taylor polarizer and an achromatic liquid crystal polarization rotator (Displaytech). Fluorescence from the sample is imaged onto a Quad-View imager (Photometrics) to split the fluorescence into four images, with two orthogonal polarizations and two wavelength bands. This microscope can also be used for two-colour smFP measurements and we have recently added the option of using a Dual-View imager (Photometrics) in experiments where polarisation measurements are not required. Image acquisition is controlled using LabVIEW software and is synchronized with the switching of the polarization rotator so that a set of frames consists of a series of images with alternating excitation polarisation, giving a total of 8 channels (parallel and perpendicular polarised excitation of donor and acceptor for two FRET pairs)\(^{18}\).

3. **TIRF-Tweezers**

Optical tweezers use a highly focussed laser beam to trap and manipulate microscopic objects. This microscope is located in the same room as the five-colour tracking system (Fig. 1e) and can access combination of lasers in the OCTOPUS hub through a second patch panel in the room. Combination of a laser tweezers system with TIRF microscopy allows the simultaneous high-resolution imaging and manipulation of structures, for example within intact cells. In this microscope a 1064 nm laser is propagated through an acousto-optic deflector (AOD) to enable a fully steerable trap which is focused at the imaging plane (Fig. 3b). A custom dichroic mirror is used to reflect the 1064 nm laser light to form the optical trap, while visible wavelengths are transmitted for fluorescence imaging. A Nikon TIRF cassette enables easy manipulation of a fibre laser beam past the critical angle of total internal reflection. The fluorescence emitted is imaged through a Nikon TE-2000 microscope onto a back illuminated EMCCD camera (Andor iXon). Fig. 5 shows an example of the use of the TIRF-Tweezers microscope to manipulate polystyrene beads in live A431 epithelial cells. 250 nm beads labelled with
fluorescein were added to a culture of A431 cells. The figure shows a time series of images of a trapped bead being brought into contact with the plasma membrane of a cell (cell membranes labelled with the fluorescent probe DiI). Functionalisation of the beads could be used to enable the characterisation of specific interactions between, for example, membrane-bound receptors and their ligands.

This microscope will enable real time manipulation and force analysis of structures in biological specimens with minimal cell damage. There is capacity to upgrade the system to incorporate a supercontinuum fibre input for multiple laser excitation and splitting optics for multiple-colour imaging as described in the systems above.

4. One-photon multicolour confocal FLIM

OCTOPUS has two confocal/FLIM systems (Fig. 1d and 1e). One is a commercial microscope (Nikon, Eclipse Ti), with confocal imaging provided through a scanning unit (Nikon, D-Eclipse C1) while an alternative confocal scanning system (Becker & Hickl, DCS-120) is used for FLIM. The second is an in-house built microscope designed for simultaneous collection of FLIM and steady state confocal images in two emission channels. In this microscope the illumination delivered by the fibre is focussed through the source pinhole and collimated to a parallel beam. The beam is reflected by a dichroic beam splitter into a scanning mirror head (GSI Lumonics), then into the microscope (Zeiss, Axiovert 135) and objective (typically a Zeiss planfluor, 40× magnification, nominal N.A. 1.3). Confocal images are collected using custom data acquisition electronics and software. Fluorescence from the sample is collected by the objective and passes back through the scan head and beam splitter into the detection path. An additional dichroic beam splitter divides the fluorescence light between two detection channels, each containing a focussing lens, pinhole, and photomultiplier (PMC100-1, Becker & Hickl GmbH). FLIM data are acquired using time-correlated single photon counting electronics (SPC-730, Becker & Hickl GmbH). The microscope is designed for maximum versatility and the two channels can be configured for a number of modes of operation (e.g. FLIM at two wavelengths and FLIM and steady state confocal imaging at the same or different wavelengths).

The illumination source for both confocal/FLIM systems can be the outputs from one of the two supercontinuum laser sources (Fianium, SC450-4, 40 MHz repetition rate), which provide tuneable pulsed illumination over a wide wavelength range. Alternatively, the microscope can be used with the outputs of a Ti:Sa laser (fs Mira, Coherent), a Ti:Sa pumped OPO (ps, Coherent), the available CW lasers, and two single wavelength pulsed sources with \( \lambda = 408 \text{ nm} \) and \( \lambda = 471 \text{ nm} \) (see below).

6. Two-Photon Microscopy

Multiphoton microscopy relies on the quasi-simultaneous absorption of two or more photons by a molecule. In the area of biological imaging, the most common form of multiphoton imaging is two-photon microscopy, in which two infrared photons are absorbed by a fluorescent probe, resulting in fluorescence emission in the visible part of the spectrum. Two-photon microscopy has several advantages over single photon techniques, in particular increased depth penetration (up to 1 mm), and reduced phototoxicity due to excitation with NIR light. The inherent femtolitre excitation volume leads to pseudo-confocal imaging.

The microscope described here is a custom-built two photon system, located in the OCTOPUS hub (Fig. 1a), and designed for maximum flexibility for biological and other imaging applications. Multiphoton laser excitation is provided by a titanium sapphire laser (Mira, Coherent), tunable between 690 and 1060 nm, pumped
by a frequency doubled vanadate laser (V18, Coherent), producing 180 fs pulses at 75 MHz. The output of this laser (3 W) is used to pump a ring cavity OPO (APE, Germany) with an output of around 300 mW, wavelength from 550-650 nm, 200 fs pulses at 75 MHz. The light is focussed to a diffraction-limited spot using one of a selection of objectives, and specimens illuminated at the microscope stage of a modified Nikon TE2000-U, the beam passing through a scanning mirror system (GSI Lumonics). Fluorescence emission passes through a bandpass filter appropriate for the fluorescent probe in use. The scan is operated in the normal mode and line, frame, and pixel clock signals are generated and synchronised with a fast microchannel plate photomultiplier tube (Hamamatsu R3809U) used as the detector. FLIM images are acquired using a time-correlated single photon counting (TCSPC) PC module (SPC830, Becker and Hickl). For FLIM analysis, grey scale images (8 bit, 256 × 256 pixels) are exported from the Becker and Hickl software as bitmaps at the same pixel threshold and analysed using SPCImage software (Becker and Hickl). A thresholding function within the FLIM analysis software ensures that noncorrelating photons arriving at the detector are not included in the analysis.

C. Using OCTOPUS to investigate complex biological phenomena

To illustrate the advantages of OCTOPUS we have chosen the EGFR network as a model system. The EGFR was discovered 40 years ago. It belongs to the ErbB family of transmembrane receptor tyrosine kinases responsible for the signalling pathways leading to cell proliferation, differentiation, migration and apoptosis, and, in humans, it is implicated in the development of most solid tumours. Structurally, this receptor consists of an extracellular growth factor-binding region, a single-pass transmembrane region and a cytoplasmic domain that has tyrosine kinase activity. The binding of its cognate ligand, the epidermal growth factor (EGF), results in ligand-induced receptor dimerisation, which is thought to be a key stimulatory step leading to allosteric transactivation of the two associated intracellular EGFR kinases and signal transduction. However, it is becoming increasingly evident that other mechanisms must be fine tuning these events, and we do not yet know the nature of additional positive and negative feedback-loops regulating the EGFR network that allow functioning under perturbation. We propose that a multi-technique approach can help in this task.

1. Combined investigations of EGFR location, internalisation, and co-location with effectors and tracking

Functional hypotheses can be derived from confocal images of protein location if proteins are expressed in cells above the detection threshold (typically a few tens of thousands per cell). Fig. 6a shows the 3-D distribution of EGFR in A431 epithelial carcinoma cells, the cell line in which EGFR was first identified. The image was obtained in one of OCTOPUS’ one-photon confocal systems, and was assembled from a stack of optical sections from cells exposed to 100 nM solution of EGF labelled with Alexa 488 dye. The 488 nm line of an Ar+ laser was used for these experiments. Cell binding was at 4°C to block receptor internalisation, which is the major pathway of EGFR degradation and signalling attenuation. Under these conditions the receptors remain localised at the plasma membrane after EGF binding. When the temperature is raised to the physiological value, receptor internalisation is promoted. Fig. 6b shows images of an initial stage of receptor internalisation, in which vesicles containing EGF/EGFR complexes are internalised into the cellular cytosol. Internalisation ultimately results in receptor degradation in lysosomes or recycling. These investigations can be complemented with time course data on the acidification of endocytic vesicles and the production of Ca²⁺ second messengers collected in adjacent microscopy systems in OCTOPUS (Fig. 1b) as previously shown.
The OCTOPUS set up also allows us to complement the above investigations with the measurement of biomolecular interactions using FLIM-FRET\(^6\). An example of how FRET can help to reject false positives in interactions derived from co-localisation is shown in Fig. 6c-f. Here we interrogate whether the GTP-binding protein Rheb, also known as Ras homolog enriched in brain (RHEB)\(^3\) interacts with EGFR at any point during internalisation. Rheb functions upstream of mTOR (mammalian target of rapamycin) and is under the regulation of growth factors and the PI3 kinase/Akt pathway\(^3\). The rationale for these experiments is that both EGFR and Rheb are targeted to the endoplasmic reticulum, the former via a retrograde route from the Golgi during EGFR trafficking to the nucleus, but the molecular mechanism in this proposed model remains unexplored\(^4-6\). For these experiments A431 cells were transiently transfected with EGFP-Rheb vector. 24 h following transfection cells were stained with EGF-Alexa 546 at 37 °C. EGF/EGFR complexes were allowed to internalise for 90 min. Cells were then imaged by confocal microscopy using 488 nm laser excitation to excite EGFP-Rheb and 543 nm excitation to excite EGF-Alexa 546. Fig. 6c shows the location of Rheb fused to enhanced green fluorescent protein (EGFP). Fig. 6d shows the distribution of EGFR molecules labelled with EGF-Alexa 546. Fig. 6e shows a merged image of EGFR and Rheb, in which EGFR and Rheb appear to be partially co-localised. However, FLIM-FRET experiments show that they do not directly interact because the lifetime of the donor is not quenched in the presence of acceptor, which would be detected if the proteins were in very close proximity (<10nm)\(^4\). TCSPC lifetime images were analysed using SPCImage (Becker and Hickl).

Information on the diffusion properties of molecules, complementary to the steady state derived from ensemble imaging, can be obtained in an adjacent OCTOPUS microscope (Fig. 1b) using single molecule tracking in living cells. At the single molecule level this can be done without the need to introduce artificial synchronisation (e.g. via cell cooling). Single molecule detection in mammalian cells is often made at the basal cell membrane because TIRF illumination is required to reduce cell autofluorescence to the level where single molecule detection becomes possible. This is because TIRF creates an evanescent field on the glass coverslip to which the cells adhere, which reduces exponentially with depth penetrating <300 nm axially into the sample\(^8\). However, in some cases single molecules can also be detected in the apical membrane\(^1\).

Fig. 6g shows a typical image of EGFR molecules in the basolateral plasma membrane of A431 cells. For these experiments we exposed A431 cells to Alexa 488-conjugated Fab fragments of monoclonal antibody mAb 2E9, known not to activate EGFR\(^4\). Fig. 6h shows the resulting tracks, which describe the paths of individual receptors or receptor complexes. Supplementary movie 1 shows how the receptors are able to move independently, collide with each other, move together for a while (as in a dimer) and then come apart\(^4\). For receptors bound to activating EGF ligands tagged with Alexa 488 we found that the ligand/receptor complexes observed are more static and their movement is slower (supplementary movie 2)\(^4\). This is consistent with activated receptors being able to bind cytoskeletal structures (e.g. actin filaments)\(^4\). However, rapid receptor movements can still be observed at the leading edges of cells in filopodia\(^4\), consistent with the previously proposed retrograde receptor transport\(^5\). Spots disappear from the image abruptly (in single steps) when the fluorophores photobleach\(^1\).

2. Investigations of in situ protein structure and conformational changes
In this section we describe how the combination of multicolour FLIM-FRET and spFRET in the OCTOPUS setup allows the investigation of mechanisms that regulate the orientation of the EGFR ectodomain and the
interfaces between receptor ectodomains in ligand/receptor complexes. The extracellular region of the EGFR is comprised of four subdomains (I–IV). Crystallographic data have shown that the unliganded receptor monomer is held in a closed conformation by an intramolecular tether formed by loops in subdomains I and III47. In ligand occupied receptor dimers the intramolecular tether is broken and the receptor is opened into an extended conformation which interacts with another monomer, forming a back-to-back dimer48, 49. However, from structural data of the EGFR ectodomain alone, the conformation of the holoreceptor with respect to the membrane cannot be derived.

The orientation of EGFR in intact cells can be derived from the distance between the binding site of EGF in EGFR and the plasma membrane23. Using this method we previously showed that the ectodomain of EGFR can be aligned both parallel and perpendicular to the membrane17, 23, 50. We also showed that EGFR can interconvert between these orientations23, 26, but the mechanism regulating EGFR orientation at the plasma membrane is not yet understood. To investigate this, we used confocal FLIM-FRET to first ask whether the distance between bound EGF and the plasma membrane, and therefore the EGFR orientation, depends on the polarity of the membrane. For this, A431 cells were exposed to the lipid dialkyldihexocyanine probe DiIC18(5) (DiD; Invitrogen), that inserts itself in the plasma membrane with the chromophore lying on the outer leaflet to act as FRET acceptor. This was followed by exposure of cells to a 100 nM solution of EGF-Alexa 546 (FRET donor). Cells were fixed with 3% paraformaldehyde and imaged at room temperature. The Alexa fluorophore was excited with 540 nm pulsed laser light from a fibre-coupled OPO and donor FLIM images were recorded in the presence and absence of acceptor. To induce membrane depolarisation we used the Na+/K+-ATPase inhibitor Ouabain51. We found that membrane depolarisation did not affect the orientation of EGFR (Fig. 7a) because the ligand-membrane distance obtained in the presence of the Ouabain treatment was the same as that found in wild-type cells (~ 6 nm)23. We conclude that changes in the membrane potential are therefore not directly involved in regulating the extracellular conformation of EGFR. We next asked whether phosphorylation-dependent intracellular interactions, could be implicated in the regulation of EGFR conformation. For this we labelled A431 cells with donor and acceptor as before and intervened in EGFR signalling pathways using a combination of 2-deoxyglucose and sodium azide (DSA) to inhibit ATP production11. We found that the distance from EGF to the membrane increased to ~ 8 nm (Fig. 7b), suggesting that the intracellular domain exerts some control on extracellular conformation as previously proposed52. These results provide an example of how OCTOPUS can help in the investigation of structure-function relationships.

SpFRET can provide complementary structural information on the nature of the interfaces formed by interacting molecules. For example, many cells show the presence of ensemble FRET between EGF molecules bound to cell surface EGFR and labelled with donor and acceptor. These observations are nevertheless surprising because the distance predicted by crystallography between the two bound EGF molecules is >11 nm48. This is larger than the distances that can be detected by FRET even allowing for extreme harmonic deformations in the crystal structure of the EGFR dimer. The occurrence of FRET in cells is therefore still not understood. The mean distance between bound EGF ligands derived from FLIM- FRET is ~ 8 nm22. These ensemble data cannot, however, report whether this is the distance between two ligands in all dimers or the mean of a distribution of widely varying distances between pairs of ligands bound to different type of receptor complexes. To distinguish between these possibilities we used spFRET. Data were collected from HeLa cells labelled at 4°C with a mixture of 0.1 nM EGF-Cy3 and 0.1 nM EGF-Atto 647. Cells were fixed with 3%
paraformaldehyde and 0.5% glutaraldehyde and imaged at room temperature. We found that the ligand-ligand distance distribution includes very short (< 3 nm), medium (~ 6 nm), and long (> 8 nm) ligand-ligand separations (Fig. 7c). These FRET data were analysed as described previously, and the results are in agreement with previous results from A431 cells\(^1\). The conclusion must therefore be that the available crystallographic data only provide high resolution structures on one of the possible interfaces between receptors. The new interactions detected by spFRET provide a new perspective on the variety of receptor-receptor interfaces that can be formed.

3. Investigating multiple protein-protein interactions in live cells

The EGFR family (also known as ErbB1) also provides an example of the type of complexity encountered in the study of functional protein networks in cells. The EGFR family has evolved from one receptor/one ligand in C. elegans, through one receptor/multiple ligands in flies, to a family comprising four receptors ErbB1-4, and 13 extracellular ligands in mammals\(^2\). ErbB4 shares with EGFR recognition features for the heparin-binding EGF-like growth factor, betacellulin, and epiregulin ligands, and with ErbB3 the ability to bind four (or more) neuregulin family proteins (NRGs)\(^3\). A defining characteristic is that only two family members, ErbB1 (EGFR) and ErbB4, can bind ligands and also signal autonomously via homo-dimerisation and trans-activation of their tyrosine kinases. In contrast, ErbB2 and ErbB3 are not autonomous. ErbB2 lacks the intrinsic ability to interact with ligand, whereas the kinase of ErbB3 is defective\(^4\). ErbB2 and ErbB3 can therefore only initiate signals through hetero-dimer formation. Accounting for heterodimers, the number of possible functional inter-receptor complexes increases from four (homo-dimers) to eight.

We are ultimately interested in exploiting the ensemble/single molecule technique complementary in OCTOPUS to derive models of ligand-induced behaviour of the ErbB1-4 family during signal transduction to derive system predictions. For example, we wish to establish how receptor-receptor interactions, receptor activation and inhibition of tyrosine kinase activity on some receptor types result in gain of function by other receptors, and how this is influenced by feed-back loops and internal and external perturbations. For these investigations, we can use the OCTOPUS single molecule multicolour tracking system, for example, to follow the movements of EGFR and ErbB2 at the plasma membrane of live T47D cells, a cell model that expresses the four receptor types at a level between 10,000-30,000 receptors per cell. Fig. 8 shows single molecule images of EGFR and ErbB2 in their basal state. The receptors are labelled with 2 nM anti-EGFR affibody (Abcam) conjugated with Alexa 488, 0.1 nM and anti-ErbB2 affibody conjugated with Atto 647N for 15 minutes at 37 °C and imaged directly on the microscope at room temperature. The resulting tracks indicate that, in the basal state, EGFR displays in these cells higher mobility at the plasma membrane than ErbB2. 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.

IV. CONCLUSIONS

The ultimate goal of biological imaging is to track in real time and at high resolution the processes underlying the functioning of organisms in health and disease. No single imaging technique is able to achieve this goal, so there is an increasing requirement to apply multiple microscopy techniques to the study of biological systems.

The OCTOPUS cluster represents a new concept in large-scale, multi-user microscopy facilities. The
combination of a multi-laser hub, integrated laser interlock and control systems, and modular microscopy stations allows for the rapid delivery of tailor-made illumination to individual microscopes. The advantages of this arrangement are: **Cost-effectiveness.** Specialised, expensive laser systems can be easily shared between microscopes. For example, pulsed OPO and super-continuum sources usually used for FLIM can be used in single molecule TIRF microscopes to obtain single molecule fluorescence lifetime data. **Ease of use.** Laser illumination can be switched between microscopes using the patch panel/fibre system with minimal need for optical realignment. This is of particular value in a high-throughput, multi-user environment in which effective exploitation of the microscopes depends on minimum down-time between experiments. **Laser safety.** Users of multi-user microscopy facilities are often not laser experts. Safe use of such facilities therefore requires significant levels of user training. This is particularly the case where lasers are aligned into microscopes through free space. The use of optical fibres and an integrated interlock system in OCTOPUS allows beam paths to be almost entirely enclosed while retaining the flexibility to interchange lasers rapidly. **Parallel development of new techniques.** In user facilities there is often a conflict between the requirement to provide routine operations while at the same time develop and commission the next generation of microscopy methods. In conventional microscopy laboratories, the development of new instruments requires either the purchase of additional lasers or the withdrawal from operation of existing microscopes. The flexibility of OCTOPUS allows new equipment to be commissioned using laser radiation from the OCTOPUS hub in parallel with user operation of existing microscopes. When commissioned, the new microscope stations are easily and rapidly made available to facility users.

The OCTOPUS cluster enables a multi-technique approach, and as an example of this we have shown how OCTOPUS microscopes have been used to follow the sequence of events during the EGFR signal transduction process. The multi-technique approach has allowed us to monitor this process from the binding of ligands to receptors, the location of ligand/receptor complexes in the cell, receptor transport and clustering at the plasma membrane, interactions between receptors with other signalling partners, interaction with cytosolic components, and finally receptor internalisation and signalling attenuation. The results also demonstrate how statistically meaningful ensemble data from molecular populations can be complemented with information on fluctuation distributions and rare events, removing uncertainties from the need to synchronise populations and time averaging but without the risk of overestimating the significance of unusual behaviours.

**ACKNOWLEDGEMENTS**

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**REFERENCES**

44. See supplementary material at [URL will be inserted by AIP] for movie showing motion of EGFR labelled with fluorescent 2E9 antibody in A431 cells (basal state EGFR).
45. See supplementary material at [URL will be inserted by AIP] for movie showing motion of EGFR labelled with fluorescent EGF in A431 cells (activated EGFR).
### TABLES

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**TABLE I.** Lasers installed in the OCTOPUS cluster.

### FIGURE CAPTIONS

**FIG. 1.** Layout of the OCTOPUS facility. a) laser hub and multiphoton confocal microscopy; b) three-colour single molecule tracking and eight-channel single molecule combined spFRET and smFP; c) data processing area; d) one-photon multicolour confocal FLIM; e) one-photon multicolour confocal FLIM, five-colour single molecule tracking, and TIRF-tweezers; f) development laboratory. The red boxes on walls mark the position of the laser patch panels.

**FIG. 2.** a) SMA optical fibre patch panel; b) Magelis Human Machine Interface control panel used to control lasers in the OCTOPUS facility; c) Cerberus information display system showing the status of the facility (green boxes indicate lasers that are switched on).

**FIG. 3.** a) Optical layout of the three-colour single molecule tracking microscope showing TIRF excitation beam (blue-green), 3 colour fluorescence emission (brown), and individual fluorescence emission channels (green, orange, and red). b) Optical layout of TIRF-tweezers system showing optical trapping beam (red), TIRF excitation beam (green), and fluorescence emission (orange).
**FIG. 4.** Simultaneous 3-colour single molecule TIRF imaging of HeLa cells labelled with a) EGF-Atto 647N; b) EGF-Alexa 546; and c) EGF-Atto 488.

**FIG. 5.** OCTOPUS TIRF-tweezers for manipulation of fluorescently labelled 250 nm size polystyrene bead (488nm excitation) in live A431 mammalian cells (DiI membrane labelled). a) to d) shows approach of trapped beads in less than 2 micron steps. Arrow indicates position of trapped bead (scale bars 20 µm). TIRF provides video rate background-free imaging of sub-wavelength resolution.

**FIG. 6.** a) 3-D distribution of EGFR in A431 epithelial carcinoma cells. The image was obtained from cells exposed to 100 nM solution of EGF labelled with Alexa 488 dye from a stack of optical sections obtained by one photon confocal microscopy (scale bar 20 µm). b) High magnification confocal images showing the initial stages of receptor internalisation in A431 cells (sample preparation same as a), scale bar 5 µm). c) Confocal image showing the distribution of EGFP-Rheb in live A431 cells. d) Confocal image showing the location of Alexa 546-labelled EGF/EGFR after internalisation, and e) merged image, scale bars 10 µm). f) Donor fluorescence lifetime image of the same cells shown in c) to e) (scale bar 10 µm). The inset shows the distribution of lifetimes in the image. g) Single molecule TIRF image of EGFR molecules in the plasma membrane of A431 cells exposed to Alexa 488-conjugated Fab fragments of monoclonal antibody mAb 2E9. h) Tracks of molecules imaged in g), which describe the paths of individual receptors or receptor complexes.

**FIG. 7.** a) FRET efficiency as a function of acceptor density measured A431 cells treated with Ouabain to induce membrane depolarisation, loaded with DiD and labelled with 100 nM EGF-Alexa 546. b) Cells treated instead with 2-Deoxyglucose and Sodium Azide (DSA) to inhibit ATP production. The best fit of Monte-Carlo simulation results to the data were calculated as previously shown and are labelled with the corresponding distance of closest approach. c) Examples of single-pair FRET traces. Fluorescence intensity versus time traces of donor (green) and acceptor (red) fluorophores. Examples are shown of long ligand-ligand separation (> 8 nm) (left; low FRET, high donor emission and low acceptor emission), medium ligand-ligand separation (~ 6 nm) (centre; moderate FRET, medium emission from both donor and acceptor), and short ligand-ligand separation (< 3 nm) (right; high FRET, low donor emission and high acceptor emission).

**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-Atto647N; c) cells labelled with 2 nM anti-EGFR Affibody-Alexa 488; d and e) single molecule tracks (white) from the spots located within the boxes marked in b and c.