Membrane protein stoichiometry studied in intact mammalian cells
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Receptor membrane proteins in the plasma membranes of cells respond to extracellular chemical signals by conformational changes, spatial redistribution, and (re-)assembly into protein complexes, for example, into homodimers (pairs of the same protein type). The functional state of the proteins can be determined from information about how subunits are assembled into protein complexes, the so-called stoichiometry. Stoichiometry information, however, is generally not obtained from intact cells but from pooled material extracted from many cells, resulting in a lack of fundamental knowledge about the functioning of membrane proteins. Firstly, functional states may dramatically differ from cell to cell on account of cell heterogeneity. Secondly, extracting the membrane proteins from the plasma membrane may lead to many artefacts. Liquid scanning transmission electron microscopy (STEM) is a new technique capable of determining the locations of individual membrane proteins within the intact plasma membranes of cells in liquid. Many tens of whole cells can readily be imaged. It is possible to analyse the stoichiometry of membrane proteins in single cells while accounting for heterogenic cell populations. Liquid STEM was used to image epidermal growth factor receptors in whole COS7 cells. A study of the dimerisation of the HER2 protein in breast cancer cells revealed the presence of rare cancer cells in which HER2 was in a different functional state than in the bulk cells. Stoichiometric information about receptors is essential not only for basic science but also for biomedical application because they present many important pharmaceutical targets.

Keywords:
Mammalian cell, STEM, ESEM, liquid-phase electron microscopy, nanoparticle, protein label, epidermal growth factor receptor, HER2, breast cancer cell

Abbreviations:
STEM - scanning transmission electron microscopy; ESEM - environmental scanning electron microscopy; QD - quantum dot; Z - atomic number; EGFR - epidermal growth factor receptor; HA - hemagglutinin

Introduction
An essential aspect of communication in all life forms is how messages are interpreted and then translated into action. Cells have receptor proteins in their plasma membranes “listening” to chemical signals from the outside world. These signals consist of ligands, small molecules that bind specifically to a receptor. But how those signals are “interpreted” and lead to decisions triggering distinct signalling pathways is incompletely understood (Bessman et al., 2014; Valley et al., 2014). A key challenge for understanding the fundamentals about cell decisions involved in the interpretation of external chemical signals is that these decisions often differ from cell to cell on account of cell heterogeneity, so that even neighbouring cells of the same cell type may show different responses to the same ligand concentrations. The chemical-physical response of a cell to the binding of an external signal molecule is termed signal transduction. The first stage of signal transduction involves the binding of a ligand and the resulting response of the involved receptor protein leading to its activation. This stage is associated with dramatic spatial and temporal changes in the distribution of these membrane proteins resulting, for example, in conformational changes, re-localisation in the plasma membrane, and their assembly into protein complexes, for example, homodimers (pairs of the same receptor type). In fact, a multiple of receptors is often at play, and many cell surface receptors have been shown to redistribute into several different protein complexes, for example, heterodimers (pairs of two different receptor types), in response to ligand binding. The picture becomes even more complicated because certain receptors, so-called orphan receptors, do not need to receive a signal in order to become active. Information about the assembly of subunits into protein complexes is called stoichiometry, and its examination reveals important clues about the functional state of the proteins. However, information is lacking about the stoichiometry of membrane proteins within individual cells and about differences between cells, and in the presence of chemical signals, so that the initiating events within the plasma membrane remain unclear (Bessman et al., 2014; Valley et al., 2014).

Limited capabilities of analytical methods to study membrane proteins
The main reason behind the lack of knowledge about the early stage of signal transduction is rooted in the limitations of the used analytical methods (Yamashita et al., 2015). A key challenge to understanding the function of membrane proteins is that it is notoriously difficult to study them. The information about the stoichiometry of protein complexes is generally not obtained from intact cells but via biochemical methods involving extraction of proteins from cellular material, via X-ray crystallography of protein crystals or other technologies (Larance & Lamond, 2015; Bessman et al., 2014). Firstly, protein material is pooled from many thousands of cells and thus most knowledge about cellular function is based on population averages. Secondly, extracting the membrane proteins from the plasma membrane may lead to artefacts in conclusions about function, since the...
Table 1. Important analytical techniques used to study the functional state of proteins (stoichiometry) and their limitations (see supplementary discussion in Peckys et al., 2015).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Limitation</th>
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<tr>
<td>Biochemical methods</td>
<td>Limited to pooled cellular material, proteins do not remain in cells</td>
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<td>Light microscopy</td>
<td>Spatial resolution insufficient to directly view stoichiometry</td>
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<td>Flow cytometry</td>
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<td>Electron microscopy</td>
<td>Prone to artefacts when determining protein stoichiometry</td>
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<td>Proximity assay</td>
<td>Does not detect dimers but reflects overall protein proximity, which is</td>
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actual molecular behaviour of the receptors is not studied in a native environment (Figure 1). Protein extraction may lead to changes in folding, protein complexes that are bound together in the plasma membrane may not necessarily remain together when extracted, and finally, certain proteins may be difficult to extract.

On the other hand, state-of-the-art light microscopy techniques using intact cells are incapable of resolving endogenously (naturally) expressed membrane proteins with sufficient spatial resolution (Peckys et al., 2015; Shivanandan et al., 2014), so that often opposing observations are published (Valley et al., 2014). Forster Resonance Energy Transfer (FRET) may result in artefacts since the dimensions in protein complexes may supersede the FRET distance (Piston & Krümers, 2007). Various other indirect fluorescence techniques exist but are mostly restricted to unnatural low protein expression levels of <1 per μm² (Arant & Ulbrich, 2014). Cryo electron microscopy can be used to image proteins in an almost native environment of amorphous ice (Kourkoutis et al., 2012) but the sample preparation and microscopy is so elaborate that it is practically impossible to study whole cells let alone series of cells. Freeze fracture sample preparation in combination with immunogold labelling (Cambi & Lidke, 2012) provides protein locations in membrane patches, but it is practically impossible to obtain stoichiometric protein information since the used antibodies are too large, the cellular context gets lost, and the techniques involve delicate sample preparation.

Finally, proximity methods (Leuchowius et al., 2013; Citri & Yarden, 2006), detect if protein pairs are in vicinity but the methods do not actually measure the distances between protein subunits, so that truly occurring protein complexes cannot be separated from proteins positioned in proximity by random chance, and the methods thus trigger many false positives for the naturally occurring high protein surface densities (Moreira et al., 2013). Table 1 lists the most important analytical techniques including their limitations preventing to study HER dimerisation in cellular subpopulations.

Studying membrane receptors from cell population averages might give incorrect insights, since individual cells and even individual areas of the plasma membrane often include proteins in a different functional state than in the average (Peckys et al., 2015). Aiming for a more comprehensive picture of signal transduction, it is thus crucial to study the distinct relations between ligand exposure and receptor redistribution at the single-molecule level within the intact cell and examine multiple cells thus allowing to account for differences within cell populations.

Liquid STEM technology

A new analytical microscopy technology to study membrane proteins in intact eukaryotic cells in their native liquid environment was introduced in the last decade (de Jonge et al., 2009; de Jonge & Ross, 2011; Peckys & de Jonge, 2014a; Peckys et al., 2015). This technology, termed liquid scanning transmission electron microscopy (STEM), overcomes key limitations in the study of cellular function at the molecular level. Eukaryotic cells in liquid are placed in a microfluidic chamber enclosing the sample in the vacuum of the electron microscope, and they are then imaged with STEM (Figure 2A). In order to obtain contrast through water and cell material of several micrometres thickness, gold nanoparticles or fluorescent quantum dots (QDs) are used as specific protein labels (Dukes et al., 2010; Peckys & de Jonge, 2015; Peckys et al., 2014a) and the atomic number (Z) contrast of STEM is employed so that nanometre resolution is obtained on tagged proteins in whole eukaryotic cells in liquid (de Jonge et al., 2009). The high resolution is achieved well within the limit of radiation damage (de Jonge et al., 2009; Hermansdorfer et al., 2016). Crucial for the study of cell function is the capability to screen hundreds of cells and to investigate selected tens of cells with high spatial resolution in the range of 3 nm, this was achieved by combining fluorescence microscopy with liquid STEM and correlating the obtained information (Peckys et al., 2011; Dukes et al., 2010). It is not always necessary to enclose the cells in a microfluidic chamber. For many studies, it is sufficient to obtain information from the thin outer regions of the cells, and those can be imaged with high resolution using environmental scanning electron microscopy (ESEM) with STEM detector (see Figure 2B) (Bogner et al., 2005; Peckys et al., 2013). A third option is given by enwrapping the cells with a graphene sheet (Park et al., 2015; Wojcik et al., 2015).

An essential aspect of the liquid STEM technology is the specific labelling of membrane proteins. Several protocols were developed to achieve specific labelling of receptors, for example, the epidermal growth factor receptor (EGFR) via its ligand using gold nanoparticles of 10 nm diameter (de Jonge et al., 2009), and with QDs providing both a fluorescence signal and Z-contrast for correlative microscopy (Peckys & de Jonge, 2015; Peckys et al., 2014a; Peckys et al., 2014b; Dukes et al., 2010). Labels were also developed for another growth factor receptor, HER2 (Peckys et al., 2015), a so-called orphan receptor that does not have a ligand to be activated and often resides in the plasma membrane in active state. This receptor is of particular relevance in various types of cancer. It is overexpressed in a particularly aggressive type of...
breast cancer (Hyne & Lane, 2005; Yu & Hung, 2000). In the absence of a ligand as for EGFR, a label was developed for HER2 using an Affibody peptide with a biotinylated C-terminus (Eigenbrot et al., 2010) (Affibody AB, Sweden) conjugated to a QD via a short biotin-streptavidin bond to a QD (see Figure 3). The Affibody does not influence the behaviour of HER2 with respect to dimerisation or cellular uptake (Orlova et al., 2006; Steffen et al., 2005). The labelling procedure was carried out in two steps (Peckys & de Jonge, 2014b) in order to avoid label induced clustering (Brown & Verkade, 2010) and to obtain binding of the Affibody-QL label to HER2 in a 1:1 ratio (Peckys et al., 2015). Live cells were first incubated with the anti-HER2 Affibody, and the cells were then chemically fixed. Finally, the cells were then incubated with streptavidin QDs. This labelling strategy works in principle also for many other membrane proteins. Instead of engineering of a binding site into the protein of interest, for example, the selective Ca$^{2+}$ channel pore-forming ORAI1 protein was provided with an extracellular hemagglutinin (HA)-tag in Jurkat T-cells (Peckys et al., 2016). The HA tag was positioned in the second extracellular loop of ORAI1, between transmembrane domain 3 and 4, between amino acid 206 and 207 (Gwack et al., 2007). The HA tag insertion was shown by others not to impair the formation of functional ion channels (Quintana et al., 2011). The cells were fixed, subsequently incubated with biotinylated Anti-HA-Fab, binding in a one-to-one stoichiometry to the HA-tag, and finally labelled with streptavidin QDs.

**Imaging EGFR in COS7 cells**

Sections of text in this chapter were copied with permission from Cambridge University Press (Peckys & Jonge, 2016). To perform the experiment, COS7 fibroblast cells were grown on microchips coated with poly-L-lysine for the promotion of cell adherence, incubated with the labels, and fixed with glutaraldehyde (Ring et al., 2011; de Jonge et al., 2009). This protocol avoids the traditional preparation steps associated with electron microscopy such as sectioning, and plastic embedding or freezing (Hoenger & McIntosh, 2009) with their high risk of perturbing the original state of the cells. Instead, the protocol is comparable with that for fluorescence microscopy, where chemical fixation is used to preserve biological structures during exposure to the intense light beam needed to detect the fluorophores (Pawley, 1995; Tanaka et al., 2010). The sample was then enclosed in the microfluidic chamber for STEM (Figure 2) and images were recorded while maintaining a continuous flow of buffer. Figure 4A shows the edge of a COS7 fibroblast cell that was incubated for 5 minutes with the label. The liquid STEM spatial resolution of 4 nm was sufficient to distinguish adjacent labels, dimers, and larger clusters (see inset). The cellular material is visible as light blue shapes. The randomly distributed localization of the EGFRs over the cellular surface is consistent with this time window of incubation (Lidke et al., 2004).

To test if the labelled EGFRs were still functional after the binding of the nanoparticle label, we examined the cells for endocytosis of labelled EGFRs, a process that naturally follows after the EGFR is activated (Glenney et al.). The cells were incubated for 10 minutes with EGF-gold, washed, incubated an additional 15 minutes in buffer, then fixed. Figure 4B shows circular clusters of labels, consistent with clustering in internalised endosomes (Lidke et al., 2004). The rounded shape in the top right corner is blurred indicating a different vertical position in the sample. These experiments were carried out with STEM at an electron dose of 7×10$^2$ e/Å$^2$, only an order of magnitude larger than used for imaging frozen cells (Hoenger & Bouchet-Marquis, 2011), and an order of magnitude smaller than the dose used for STEM on conventional thin sections (Sousa et al., 2011). Signs of radiation damage, such as displacement or shifting of the EGFR-bound AuNPs in subsequently recorded images, only appeared after several image exposures. The sample fixation thus provided sufficient stability to the sample, and the liquid flow presumably also helped to reduce the damaging effects from electron beam induced radicals, free electrons, and heat (de Jonge et al., 2009). The liquid thicknesses were in the micrometres range, as measured from the total number of electrons.
scattered towards the annular dark field detector. The spatial resolution achieved is remarkably high, and is due to the Z contrast of STEM (de Jonge et al., 2010; Demers et al., 2010; Schuh & de Jonge, 2014). It would not be possible to achieve nanoscale resolution at these thicknesses with transmission electron microscopy. Furthermore, although it has been suggested that Brownian motion should blur the images, this is not seen experimentally; instead, nanoparticles in close proximity to a membrane move several orders of magnitude slower than what would be expected for a bulk liquid (Ring & de Jonge, 2012; White et al., 2012; Verch et al., 2015).

Indeed, atomic resolution has been reported for TEM in liquid (Yuk et al., 2012; Evans et al., 2011). Nanoparticle labels appear to be sufficiently immobilised in live or fixed cellular structures to allow nanoscale microscopy (Peckys et al., 2011; de Jonge et al., 2009).

**Studying HER2 in intact breast cancer cells**

With liquid STEM it is feasible to collect data on receptor membrane expression and stoichiometry in single cells. The results shown in Figure 5 demonstrate the usage of the analytical technique for research on HER2 in cancer cells. SKBR3 cells, a HER2 overexpressing human breast cancer cell line, were studied with correlative fluorescence microscopy and liquid ESEM-STEM (Peckys et al., 2015). (A) Fluorescence overview image showing several dozens of cells. Individual cells exhibit a high degree of heterogeneity in their morphology and HER2 membrane expression. (B) Fluorescence image of the cells within the boxed area in A. (C) Liquid STEM image of the boxed region in B recorded at 15,000 × magnification using ESEM. (D) STEM image recorded in the boxed region shown in C at 75,000 × magnification. The locations of individual HER2 receptors labelled with QDs are visible as the bright spots. The brighter background features represent membrane ruffles. Many pairs (homodimers) are visible; two are indicated with the arrowheads. Note that the image looks very different to conventional electron microscopy images showing the cellular ultrastructure. From (Peckys et al., 2015).

Figure 6. Statistical analysis of the spatial distribution of labelled HER2 proteins in eleven SKBR3 cells using the pair correlation function g(r) (Peckys et al., 2015). (A) g(r) calculated for a total of 14,171 labels exhibited a peak at 20 nm indicating HER2 dimerization. Larger-sized clusters were also observed. (B) HER2 pairs were absent in cellular areas with homogeneous or flat membrane topology (3,307 labels), contrasting g(r) in the ruffled areas. (C) HER2 was only clustered in the two analysed flat cells (3,864 labels). Clustering was only observed in cells with membrane ruffles. From (Peckys et al., 2015).

In contrast to conventional electron microscopy studies, the cells were imaged as a whole and in liquid state, so that the membrane proteins remained in the intact plasma membrane. The locations of individual HER2 receptors were detected using an anti-HER2-Affibody in combination with a QD label. Fluorescence microscopy revealed considerable differences of HER2 membrane expression between individual cells, and between different membrane regions of the same cell (Figure 4A). Subsequent ESEM of the corresponding cellular regions provided images of individually labelled HER2 receptors (Figure 4D). The high spatial resolution of 3 nm, the 1:1 labelling stoichiometry, and the close proximity between the QD and the receptor allowed quantification of the stoichiometry of HER2 complexes. It was also possible to distinguish between monomers, dimers, and higher order clusters.

The clustering behaviour of HER2 was statistically analysed via the pair correlation function g(r) (Stoyan & Stoyan, 1996), calculated from all individual HER2 positions. These positions were automatically detected using a software tool designed by our group for this purpose. The function g(r) measures the likelihood of a particle to be found within a certain radial distance with respect to a reference particle, whereby g(r) = 1 represents a random distribution, and a value >1 indicates clustering with a higher probability than random occurrence (Stoyan & Stoyan, 1996). In measurements incorporating 14,043 HER2 positions in eleven cells, a sharp peak in the g(r) function at 20 nm indicated that HER2 was clustered as a homodimer (Figure 5A). A centre-to-centre label distance of 20 nm was expected on account of the size of the HER2 dimer, and the two quantum dot labels (Peckys et al., 2015).

HER2 distribution patterns were determined for two distinct cellular regions: membrane ruffles and homogeneous or flat areas. A remarkable difference was found from analysing g(r) for these different membrane regions. HER2 homodimers (peak at g(r) = 20 nm) appeared in ruffled regions but were entirely absent from homogeneous membrane regions (Figure 5B). In cancer cells, the highly dynamic membrane ruffles, also referred to as invadopodia, are considered to serve as junctions for cellular signalling, and drive motility, invasiveness, and metastasis of cancer cells (Weaver, 2006; Feldner & Brandt, 2002; Brix et al., 2014). The results could thus imply that HER2 homodimers play a role in cancer cell spreading, which is supported by the findings of others showing that HER2 overexpression increases the oncogenic potential in breast epithelial cells (Inghotthson et al., 2015).

A second imperative finding was the discovery of a small subpopulation of cells with a different phenotype than the average cell (Peckys et al., 2015). This group of cells was characterised by flat peripheral membrane regions and can possibly be identified as resting (possibly dormant) cells. HER2 homodimers were found to be absent from this subpopulation of cells (Figure 5C), even though the concentration of HER2 in the plasma membrane was only ~30% lower than in the bulk cancer cells. The absence of HER2 homodimers from these flat cells likely indicates a different intercellular signalling mechanism than the average/bulk SKBR3 cell.

**Conclusions**

Liquid STEM is a new electron microscopy technology with the unique capability to measure the individual locations of endogenous proteins within the intact plasma membranes of cells, combined with the capability to screen hundreds of cells with correlated light microscopy. It enables the analysis of the functional (stoichiometric) state of membrane proteins at the molecular level in single cells and can account for heterogenic cell populations by examining tens to hundreds of individual cells. A spatial resolution of 3 nm is achievable on labelled proteins in whole cells and within the limit of radiation damage. The sample preparation is similar to that for fluorescence microscopy, and it is readily possible to examine many tens of cells. Although the role of HER proteins is an important topic in cancer...
research, which has been extensively explored using a wide range of techniques, the aforementioned presence of rare HER2 overexpressing cancer cells, in which signalling active HER2 homodimers were absent, was not unveiled before. The findings are not only relevant for the basic science behind this receptor, but also for research on the mechanism behind drug resistance development, which is presumably rooted in cancer cell heterogeneity (Bedard et al., 2013).

**Prof. Dr. Niels de Jonge**

Prof. Dr. Niels de Jonge received his M. Sc. in Physics from the University of Freiburg, Germany (1999). He worked as senior scientist at Philips Research, Netherlands, on carbon nanotube electron sources (2000-2005). He was strategic hire at Oak Ridge National Laboratory (ORNL), where he pioneered scanning transmission electron microscopy (STEM) of liquid specimens (2005-2010). He joined Vanderbilt University School of Medicine, as Assistant Professor of Biophysics (2007-2011). He is currently senior group leader at the INM – Leibniz Institute for New Materials, Germany (2012 - present), and Honorary Professor of Experimental Physics, Saarland University, Germany. His main research area is in situ electron microscopy of biological samples in liquid phase, for example, to study membrane protein complexes in cancer cells. He received the ORNL innovation award, the ORNL Esprit de Corps award, the 2016 MRS Innovations in Materials Characterization Award, and the 2016 European Microscopy Award life sciences category.

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**References**


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