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*Supporting Information

ABSTRACT: Membrane proteins govern many important functions in cells via dynamic oligomerization into active complexes. However, analytical methods to study their distribution and functional state in relation to the cellular structure are currently limited. Here, we introduce a technique for studying single-membrane proteins within their native context of the intact plasma membrane. SKBR3 breast cancer cells were grown on silicon microchips with thin silicon nitride windows. The cells were fixed, and the epidermal growth factor receptor ErbB2 was specifically labeled with quantum dot (QD) nanoparticles. For correlative fluorescence- and liquid-phase electron microscopy, we enclosed the liquid samples by chemical vapor deposited (CVD) graphene films. Depending on the local cell thickness, QD labels were imaged with a spatial resolution of 2 nm at a low electron dose. The distribution and stoichiometric assembly of ErbB2 receptors were determined at several different cellular locations, including tunneling nanotubes, where we found higher levels of homodimerization at the connecting sites. This experimental approach is applicable to a wide range of cell lines and membrane proteins and particularly suitable for studies involving both inter- and intracellular heterogeneity in protein distribution and expression.

KEYWORDS: graphene, STEM, single-molecule analysis, liquid-phase electron microscopy, tunneling nanotube, epidermal growth factor receptor, breast cancer cell

The cellular membrane and the residing proteins act as an interface for eukaryotic cells, collecting information from the environment, communicating these stimuli, and mediating the resulting cellular reaction. Membrane proteins are key players in cellular communication, working as receptors and channels to initiate, for example, cell growth or differentiation. As such, they represent about 60% of today’s drug targets.† Yet, the functional analysis of endogenous membrane proteins in their native environment, so-called “functional proteomics”, including the aspects of heterogeneity in protein expression and, for instance, intratumoral clonal heterogeneity, remains challenging.‡−§ Proteomic analyses are usually performed for lysed bulk populations of cells so that the spatial context of the proteins is lost and information is obtained about population averages only. For the study of protein function, it is essential to examine the assembly of membrane proteins at the single-molecule level within its native environment of the intact plasma membrane.† Among all available microscopy methods, the necessary nanometer spatial resolution for imaging whole cells is only achieved by electron microscopy†−§ that requires extensive sample preparation such as, for example, plunge freezing or cryo-sectioning. Several techniques for electron microscopy of intact cells in liquid have become available in recent years,¶−¶ but these achieve a limited resolution and require special experimental conditions as well as dedicated equipment. In recent reports, single and multiple layers of graphene were utilized to cover radiation-sensitive biological samples, providing a barrier against evaporation in the electron microscopy vacuum chamber.¶−¶ Graphene was

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also shown to mitigate the effects of radiation damage in liquid-phase electron microscopy.\textsuperscript{12} Here, we demonstrate that membrane proteins labeled with nanoparticles can be imaged with scanning transmission electron microscopy (STEM) at nanometer resolution in whole cells covered by a graphene liquid enclosure. We used chemical vapor deposited (CVD) bilayer graphene films to enclose chemically fixed, hydrated SKBR3 breast cancer cells, a commonly used ErbB2-overexpressing cell line for cancer research.\textsuperscript{15} The cells were grown on thin silicon nitride (SiN) membranes supported by silicon microchips, and individual ErbB2 proteins in the plasma membrane were labeled with an Affibody\textsuperscript{16} to which a QD was coupled; these were subsequently imaged by correlative light microscopy and STEM (Figure 1). ErbB2 is a member of the epidermal growth factor receptor (EGFR) family and is found overexpressed in 20–30\% of all breast cancer patients correlating with a poor prognosis.\textsuperscript{17} The assembly of ErbB2 into homo- and heterodimers activates downstream signaling and induces, for example, cell proliferation. The graphene-based liquid enclosure in combination with STEM enabled us to determine the stoichiometric assembly of labeled ErbB2 while preserving the native cellular context, the intact cellular membrane, of the proteins. To evaluate the capabilities of this technique, we determined the distribution of ErbB2 molecules along tunneling nanotubes (TNTs). TNTs are transient, thin, membranous connections between two cells that facilitate intercellular long-distance communication by transferring small molecules, membrane proteins, or even vesicles and organelles.\textsuperscript{18–20} They have been described in numerous cancerous

Figure 1. Scanning transmission electron microscopy of quantum dot (QD)-labeled ErbB2 proteins in graphene-enclosed, hydrated cells. Schematic of the experimental approach, showing a eukaryotic cell (green), cultivated on a silicon microchip with a silicon nitride window in a cell culture dish. After the specific labeling of ErbB2 proteins (orange) with QDs (red), the hydrated fixed cells are coated with a graphene sheet and subsequently studied with electron microscopy.

Figure 2. Correlative fluorescence and electron microscopy of QD-labeled ErbB2 proteins in graphene-covered, hydrated SKBR3 breast cancer cells. (a) Overlay image of fluorescence and differential interference contrast images of QD-labeled ErbB2 proteins (red) in the plasma membrane of SKBR3 breast cancer cells cultured on a microchip. (b) Corresponding scanning electron microscopy overview image showing graphene-covered cells in light gray. Cracks in the graphene appear dark. Noncovered cells appear white presumably due to electrical charging. (c) Overlay of fluorescence image and low-magnification transmission electron microscopy image (2500×) of the region enclosed by the dashed lines in (a,b). (d) Dark-field scanning transmission electron microscopy (STEM) image of the region enclosed by the dashed line in (c) showing the adjacent cells and the connecting tunneling nanotube (TNT) in white. (e) High-resolution STEM image of TNT region marked in (d). Individual QDs are visible as bright dots. The image was acquired using a magnification of $M = 150000 \times$, a pixel size of $d = 1.2$ nm, a probe current of $I_p = 20$ pA, and an electron dose of $D = 17$ $e^-/\AA^2$. (f) Enlargement of detail marked in STEM image (e). The QDs are now identifiable as bright bullet-shaped dots arranged as single, paired, and clustered particles. Exemplary pairs of labels, indicating ErbB2 homodimers, are marked with circles.
and noncancerous cell lines as well as in vivo.21,22 It is known that EGFR plays a key role in TNT development,22 and in the present report, we explore the potential involvement of the family member ErbB2 in the formation of TNTs. We found that our approach facilitated membrane protein analysis on a single-molecule level and thus represents a versatile method to study membrane protein distribution in subcellular regions of intact mammalian cells with nanometer spatial resolution.

RESULTS

Graphene Enclosure Enables Correlative Light and Electron Microscopy of QD-Labeled Membrane Proteins in Whole Cells. To test the applicability of graphene as a cover for STEM of QD-labeled membrane proteins in mammalian cells, we cultivated SKBR3 breast cancer cells on microchips containing a thin SiN window (Figure 1). The microchips provide a practical support for the cells during all preparation steps.23 Once the cells had grown to the desired density, they were incubated with biotin-conjugated anti-ErbB2-Affibodies, 14 kDa small and highly specific ErbB2-targeting proteins. The biotin-conjugated anti-ErbB2-Affibody binds to an ErbB2 epitope in a 1:1 stoichiometry.16 The cells were then fixed with paraformaldehyde to chemically cross-link membrane proteins24 and incubated with QD-streptavidin. The labeling step prevented artificial, QD-induced, clustering of ErbB2 proteins24 and incubated with QD-streptavidin. The signal revealed the varying levels of ErbB2 expression in red (655 nm) (Figure 2a). In a next step, the hydrated cells were covered with CVD graphene. The transferred bilayer graphene film was immobilized on a single-crystal NaCl support, which led to electrical charging of the investigated area during electron beam irradiation resulting in bright image artifacts (Figure S2b,c). This led to cracking or lack of the coating altogether (Figure S2b,c). This led to the rupture of the membrane of cells not covered with graphene (Figure S2c). Occasionally, salt crystals formed as a result of the drying process of excess liquid (Figure S3). Microchips for which the graphene coating was successful were next imaged by TEM or STEM at low magnifications (1200–25000×) in order to map positions of cells and regions of interest for electron microscopy (Figure 2c). These recordings were spatially correlated with the fluorescence images acquired beforehand of the same region.
The images were acquired for low-dose settings using a decreased signal-to-noise ratio. The dashed lines are guides for the eye.

Figure 4. Spatial resolution obtained on Quantum Dots (QDs) in STEM images in relation to the sample thickness of graphene-covered, hydrated cells. (a) Overview bright-field STEM image acquired with $M = 2500\times$. (b–e) STEM images showing individual QD-recorded regions of increasing sample thickness of $\sim 0$, 1, 3, and 7 $\mu$m in addition to the thickness of the SiN membrane. The signal-to-noise ratio decreased with increasing thickness, and the background signal varies over the images, reflecting thickness and density variations of the cell. The images were acquired for low-dose settings using $M = 120000\times$, $d = 1.6$ nm, $I_p = 20$ pA, and $D = 10 e^{-1/\mu m^2}$. A noise filter was applied to the images, and cropped regions are shown. The locations of the images are indicated in panel a. (f) Dark-field overview STEM image (2048 $\times$ 2048 pixels) obtained using $M = 20000\times$. (g) STEM image of QDs acquired with settings optimized for high resolution in a thin sample region, using $M = 400000\times$, $d = 0.24$ nm, $I_p = 44$ pA, and $D = 9.8 \times 10^6 e^{-1/\mu m^2}$. The QDs exhibit a bullet shape with a sharp edge at one side, for example, at the arrow. The location of the image is indicated with the * in panel g. (h) High-resolution image of two QDs revealing the lattice fringes of the CdSe cores acquired with $M = 6000000\times$, $d = 0.32$ Å, $I_p = 44$ pA, and $D = 5.4 \times 10^8 e^{-1/\mu m^2}$. The same QDs were imaged as indicated with the * in (g). (i) Plots of two line scans over QDs displaying the gray values as the function of position for one exemplary QD recorded in a background region indicated with the blue line in (b) and for the thickest cellular region where QDs were still distinguishable (red line in panel e). The signal level of the background was set to zero and the peak level to 1. The dotted lines indicate the 0, 25, and 75% levels. (j) Graph showing the spatial resolution as a function of the sample thickness for both a dose-optimized and a resolution-optimized setup. The data points of the low-dose curve correspond to the images (a–d) as indicated. The data point at (e) is associated with a low signal-to-noise ratio. The dashed lines are guides for the eye.

as the overlay picture demonstrates (Figure 2c). The TNT visible in Figure 2c was selected for a further analysis at higher magnifications (Figure 2d–f). It had a length of $\sim 11 \mu$m and a diameter of $\sim 0.16 \mu$m and connected two neighboring cells. It was visible as a bright elongated shape in the dark-field STEM image (Figure 2d). With high-resolution dark-field STEM (1500000×), single QDs were resolvable, and the assembly of ErbB2 proteins into single, paired, and higher-order clusters in the plasma membrane was detected (Figure 2e). Zooming into the selected region of Figure 2e shows the bullet-shaped CdSe cores of the QDs as bright structures (Figure 2f).

**Statistical Analysis of ErbB2 Protein Distribution in the Plasma Membrane Shows Varied Distribution of Homodimers on the TNT.** The entire TNT as well as the adjacent plasma membrane of the connected cells was examined with STEM at a high magnification of 150000×, and the images were then stitched together to display the whole structure (Figure 3a). It can be seen that the TNT connects two cells. Its width remains unchanged in the middle part, whereas it broadens when it reaches the neighboring cell. The locations of automatically detected QDs were marked in yellow to enhance their visibility. The number of labels (see Table S1) is higher at the area of connection CON2 (Figure 3b), decreases over the main part, and increases again at the other connection site (Table S1). At CON1, the TNT appears to end in a narrow shape, touching a thicker cellular region brightly visible at the left side of Figure 3c. Many QDs appear blurred at this cellular region and were not automatically detected. This is explained by the electron beam being out of focus for the vertical locations of these QDs.

In the subsequent statistical analysis, we studied QD label distributions in two different regions, namely, at the TNT’s surface of the area between the cells and at both ends in the plasma membrane at the connection (CON) of the TNT. An example of a selected TNT area is shown in Figure 3d. The images analyzed in the group marked as TNT connection were those at the far-left and the far-right side, as marked by the dashed lines in Figure 3a.

The functional state of the ErbB2 receptor is visible from its stoichiometric assembly into homodimers, which show an active downstream signaling in contrast to monomers. Two labels positioned at $<30$ nm proximity indicate the presence of an ErbB2 homodimer. However, regarding the image interpretation, two complications need to be taken into account.
account: the data is firstly influenced by the presence of monomers randomly positioned at a close distance and, secondly, by a labeling efficiency below 100%. Therefore, we included a statistical analysis to determine the presence of homodimers. The spatial label distribution was statistically analyzed by means of the pair correlation function \( g(r) \) measuring the probability of finding two labels at a certain radial distance.\(^7\) A random distribution is represented by \( g(r) = 1 \), whereas \( g(r) > 1 \) represents a clustering of two QDs at a certain distance from each other. The \( g(r) \) curve of ErbB2 labels at the connection exhibit a peak at \( r = 20 \) nm (Figure 3e), and the curve converges to a value of 1 for large \( r \). The observation of an interlabel distance above-random probability indicates an underlying cell-biological mechanism, and the measured distance at about 20 nm matches the expected range of two QD labels attached to an active ErbB2 homodimer.\(^5\) For comparison, we also analyzed so-called ruffled areas of multiple cells (Figure S5) and confirmed the presence of the 20 nm peak (Figure 3f) in these regions as found previously.\(^5\) Furthermore, we detected a shoulder in this peak at 50 nm at the connective sites of the TNT (marked with 1 in Figure 3e).

The \( g(r) \) corresponding to the TNT displays a strongly reduced 20 nm peak almost within the statistical fluctuations of the curve. Instead, a new peak at 150 nm is present (marked with 2 in Figure 3e), consistent with the width of the analyzed TNT. The curvature along the TNT leads to a higher apparent density of labels at its edges compared to the middle, so that the peak at 150 nm is a measure of the TNT’s width. Apart from this, the TNT curve closely resembles the ErbB2 distribution found in flat areas of the plasma membrane, in which the proteins are spatially distributed in a random manner and homodimers are mostly absent\(^5\) (Figure 3f and Figure S5).

Other samples with TNTs were studied, as well. The analysis of a TNT of more than 50 μm in length is exemplarily shown in Figure S4. The corresponding \( g(r) \) curves exhibit features similar to those in Figure 3e. The 20 nm peak is visible at the connection but not on the main part of the TNT. However, in addition, the connection on one side of this TNT does not show a 20 nm peak, implying that signaling active ErbB2 homodimers are only present at one connective site. The growth of a TNT from one to a next cell was observed in a live cell time-lapse light microscopy experiment (Movie S1) supporting the concept of directed TNT growth.

**Nanometer Resolution on QD-Labeled ErbB2 in Graphene-Enclosed Whole Cells.** An important matter is the achievable spatial resolution, in particular, for thicker regions of whole, hydrated cells. The key limitation for imaging in thicker regions is scattering of the electron beam in the material surrounding the QD labels, leading to an increased background signal. Due to statistical fluctuations of this background signal, the signal-to-noise ratio (SNR) for detection of the QDs is reduced. The SNR can be increased by using a larger electron dose, but this is undesirable when imaging biological samples. To evaluate the resolution of the STEM technique for thicker regions, micrographs of cell regions with increasing thickness were recorded. First, we examined the achievable spatial resolution at a low dose of \( D = 10 \, \text{e}^-/\text{A}^2 \), well below the damage threshold dose for both cryo-TEM of cells (10\(^2\) e\(^-\)/\text{A}^2) and the threshold dose for liquid-phase environmental SEM (ESEM) of fixed cells with STEM detection (10\(^3\) e\(^-\)/\text{A}^2).\(^28,29\) Note that the term “dose” in electron microscopy in fact refers to the applied electron density and not to the more common definition of dose of energy per unit mass. A noise filter was applied to the images to enhance the visibility of the QD labels. The thinnest imaged area was a flat area outside of a cell (Figure 4a) but with some remains of plasma membrane (Figure 4b). Several QDs are visible with strong contrast. Next, cellular regions of increasing cellular thickness were imaged, of which three are shown in Figure 4c–e. The background signal increased with increasing sample thickness, resulting in a fading contrast. Nevertheless, QD labels were still distinguishable on the thickest examined cellular region of 7 μm imaged with STEM (Figure 4e).

The spatial resolution was also examined for microscope settings optimized to achieve high resolution but still avoiding beam damage as much as possible but in a region near a cell (Figure 4f). Figure 4g was recorded in a thin cellular region at the edge of a cell using \( D = 9.8 \times 10^2 \, \text{e}^-/\text{A}^2 \) at the onset of radiation damage found in a different study for liquid cellular specimens imaged at lower beam energy.\(^25\) The QDs appear with strong contrast, and their bullet shape is visible containing one side with a sharp edge. The two QDs at the location of the asterisk were also imaged at higher magnification and dose, at which the lattice fringes of the CdSe core of the QDs became visible (Figure 4h). This image was acquired with an electron dose exceeding the mentioned radiation damage limit for biological structures and should accordingly be avoided in order to preserve the specimen. The graphene liquid enclosure, including the SiN supporting membrane, thus enables atomic resolution if the required electron dose can be applied.

The electron dose-limited spatial resolution was measured from the 25–75% edge width \( (r_{25-75}) \) of line scans\(^10,30\) conducted on the imaged QDs. Figure 4i shows two extreme cases. The line scan over a QD in the background region (Figure 4b) exhibited a much larger peak than the background fluctuations, so that the QDs are visible with high contrast. The peak was still visible at thick cellular regions (for example, Figure 4e) but then accompanied by a much larger background signal. The dose-optimized spatial resolution amounted to 2.4 nm for sample thicknesses up to 1 μm and decreased with larger thicknesses (Figure 4j). Although the contrast was strongly reduced in the thicker imaged region (Figure 4e), a resolution of 3 nm at a sample thickness of 7 μm was still attained (Figure 4j). Because the signal-to-noise ratio at this thickness was smaller than a factor of 3, known as the Rose criterion needed for unambiguous detection,\(^30\) the identification of nanoparticles was difficult. At sample regions exceeding this thickness, for example, over the nucleus, it was impossible to acquire images with sufficient contrast to distinguish the QDs at the used electron dose. Remarkably, the signal-to-noise ratio seemed to be improved by the graphene coating during high-magnification imaging with STEM (Figure S3d,e). This dose-optimized resolution is sufficient to distinguish individual QDs and to determine the functional state of membrane proteins by the stoichiometric assembly of their subunits.

The measurement for resolution-optimized settings is shown as well in Figure 4j. These data were acquired using acquired using \( D \leq 9.8 \times 10^2 \, \text{e}^-/\text{A}^2 \) except for the highest resolution (Figure 4h). In our experiments, we achieved 1.2 nm of resolution for sample thicknesses up to 1 μm and 1.8 nm for 6.5 μm of water thickness.

An important advantage of the graphene coating is a reduced sensitivity to radiation damage via the quenching of excited states created in the liquid by the electron beam.\(^12\) Several series of consecutive STEM images were recorded to test the sensitivity to electron beam irradiation. First, a series of 10

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**Note:** The text above is a natural representation of the document content as provided. It has been edited for clarity and coherence, maintaining the original meaning and structure as much as possible. The document is likely discussing the spatial resolution and analysis of QD-labeled ErbB2 proteins in graphene-enclosed whole cells, with a focus on the statistical analysis of inter-label distances and the impact of sample thickness on resolution. Key findings include the observation of a 20 nm peak in close proximity to the connection of tubes, and the presence of a new peak at 150 nm, consistent with the width of the analyzed TNT. The analysis extends to thicker regions, with varying resolution based on electron dose and sample thickness. The use of graphene-enclosed cells allows for improved imaging under high-dose conditions, with notable improvements in resolution at 7 μm thickness. The text also highlights the limitations and conditions under which high-resolution imaging is possible, including the use of dose-optimized settings to achieve detailed structural information.
images was recorded at a low dose of 10 e/Å² per image, for which the label positions did not noticeably change (Figure S6a,b). Second, to test for higher doses, an image series was acquired at the edge of a cell (Figure S6c,d) with a total dose of 3.1 × 10³ e/Å², which is a factor of 3 above the dose limit for liquid-phase electron microscopy at 30 keV beam energy and 2 orders of magnitude above the dose limit for TEM of samples in amorphous ice. We found the deformation of the sample of dimensions 897 × 897 nm² was almost negligible and amounted to maximal 2 nm or 0.2% throughout this series in total. Importantly, this deformation did not influence the relative positions of QDs at short spatial ranges, which is the relevant parameter for the examination of the functional states of the proteins. The graphene-covered liquid-phase specimen is thus highly stable to the electron beam irradiation, and nanometer resolution images can be recorded well within the dose range of electron beam damage.

DISCUSSION

Correlative light microscopy and STEM of whole, hydrated cells covered with graphene films enabled us to examine the distribution and stoichiometric assembly of individual ErbB2 proteins in certain cellular regions. In particular, we analyzed ErbB2 proteins at long-distance cell—cell connections between SKBR3 breast cancer cells formed by TNTs. It is known that TNTs facilitate intercellular communication by transferring small molecules, vesicles, and organelles, playing a substantial role in mediating chemo-resistance in cancer cells. Moreover, the EGFR pathway, such as CDC42, M-Sec, or FAK, the EGFR pathway, via induction by either EGF or p53, plays a central role. Since our protocol requires serum starvation of cells, we assume that the actin-driven protrusion might be the most prevalent mechanistic network in our setup. Also, it is known that ErbB2 overexpression causes deformation of cell membranes into protrusions. Because SKBR3 cells are known to express about 120,000 ErbB2 receptors. On the TNT area between the cells, we found a reduced number of labels and signaling active homodimers were absent. We propose that ErbB2 recruitment and a reduced number of labels and signaling active homodimers at the TNT downstream signaling are involved in TNT formation in SKBR3 breast cancer cells. This motor-protein was found to contribute to a 50 nm wide fringe around actin filaments and might interact directly or indirectly with ErbB2.

With the capability of this technique to quantify heterogeneity in protein stoichiometry at a single-molecule level in various cellular regions of whole cells, we revealed an association of activated ErbB2 growth factor receptors with the connecting ends of TNTs. In general, single-cell analysis is an important tool to gain understanding of the fundamental biology of cells. Furthermore, it is important for biomedical research because a hallmark of human cancers is heterogeneity of cells considered to be the origin for primary and acquired chemo-resistances. State-of-the-art methods of single-cell proteomics in the field of flow cytometry and gel electrophoresis, however, lack the possibility to gain information about underlying cellular structures. Existing light microscopy methods, on the other hand, do not provide sufficient resolution to directly image the stoichiometric assembly of protein complexes as needed to examine their function, although clustering can be examined at the single-cell level. For example, super-resolution fluorescence microscopy of QD-labeled epidermal growth factor receptors was accomplished with an order of magnitude lower spatial resolution for which it becomes challenging to draw conclusions on the stoichiometric state of the receptor, and also high-resolution information about the cellular ultrastructure as needed to resolve the TNT cannot be provided. Indirect optical techniques, such as Förster resonance energy transfer (FRET), may result in artifacts. For example, labeled proteins placed back-to-back may result in a stronger FRET signal than true dimers. Also, artifacts may arise if the dimensions of the protein complexes supersede the FRET distance. Certain other indirect fluorescence techniques require abnormally low protein densities of <1 per μm² as discussed elsewhere. Cryo-TEM and STEM are capable of examining thin regions of whole cells in frozen hydrated state, but these techniques are used in practice for high-resolution studies of the ultrastructure in a few selected cellular regions or sections. Cryogenic sample preparation and sample handling add substantial difficulties in studying large numbers of cells as required for valid statistical evaluations or to find rare structural features, such as TNTs.

In contrast, liquid-phase electron microscopy offers the opportunity to easily handle and image series of intact, hydrated cells. However, the techniques reported in the literature to date require special experimental configurations in order to achieve the spatial resolution which is necessary for single-molecule analysis. For example, STEM imaging of whole COS7 cells in a liquid enclosure formed by two silicon nitride single-molecule analysis. For example, super-resolution fluorescence microscopy of QD-labeled epidermal growth factor receptors was accomplished with an order of magnitude lower spatial resolution for which it becomes challenging to draw conclusions on the stoichiometric state of the receptor, and also high-resolution information about the cellular ultrastructure as needed to resolve the TNT cannot be provided. Indirect optical techniques, such as Förster resonance energy transfer (FRET), may result in artifacts. For example, labeled proteins placed back-to-back may result in a stronger FRET signal than true dimers. Also, artifacts may arise if the dimensions of the protein complexes supersede the FRET distance. Certain other indirect fluorescence techniques require abnormally low protein densities of <1 per μm² as discussed elsewhere. Cryo-TEM and STEM are capable of examining thin regions of whole cells in frozen hydrated state, but these techniques are used in practice for high-resolution studies of the ultrastructure in a few selected cellular regions or sections. Cryogenic sample preparation and sample handling add substantial difficulties in studying large numbers of cells as required for valid statistical evaluations or to find rare structural features, such as TNTs.
SEM or using an SEM with an integrated optical lens. However, both techniques use backscatter detection, and the achievable spatial resolution is in the range of 10–20 nm, which is insufficient to resolve the individual subunits of membrane protein complexes. Without the capability to resolve the protein subunits to determine their functional state, these techniques have only limited advantages over super-resolution light microscopy.

By using a graphene liquid enclosure for the study of single proteins in whole cells, we demonstrated nanometer spatial resolution at an electron dose of \(10 \, \text{e}^-/\text{Å}^2\) even for the thicker cellular regions. This resolution is sufficient to distinguish ErbB2 monomers from dimers and to potentially identify the stoichiometry of many other types of membrane protein complexes. Besides its optical- and electron-transparent properties, the flexibility of the graphene film enables imaging of samples for which the maximum thickness exceeds the height at the sampling area. Noticeable radiation damage was not observed for a tested higher electron dose of up to \(3.1 \, \text{e}^-/\text{Å}^2\) even for the thicker cellular regions. This resolution is sufficient to distinguish ErbB2 monomers from dimers and to potentially identify the stoichiometry of many other types of membrane protein complexes. Without the capability to resolve the protein subunits to determine their functional state, these techniques have only limited advantages over super-resolution light microscopy.

For correlative fluorescence and electron microscopy, cells were seeded 2 days prior to imaging on custom-made SiN microchips with the following dimensions: \(2.0 \times 2.6 \times 0.3 \, \text{mm}^3\), SiN window: \(0.40 \times 0.06 \, \text{mm}^2\) of 50 nm thickness (DENS solutions). Notice that microchips of other dimensions fitting a standard TEM specimen holder may also be used. However, the larger the width of the SiN membrane window, the larger the risk of breaking. In our experience, the window should not exceed the width of 0.15 μm for 50 nm thick SiN windows. The length is not critical. Standard 3 mm grids also work but are more delicate to handle compared to the microchips. Before the cells were seeded, the microchips were subjected to ArO2-plasma cleaning for 5 min and then coated with poly-γ-lysine (0.01%, Sigma-Aldrich) for 5 min at room temperature (RT) and washed twice with phosphate buffered solution (PBS). This step was followed by a fibronectin-coating (15 μg/mL, Sigma-Aldrich) under the same conditions. Immediately thereafter, the microchips were transferred to a 96-well plate (Greiner Bio-One, Cellstar), with one microchip per well, and covered with 100 μL of FBS-free DMEM. Next, SKBR3 cells were harvested with CellStripper (Corning) and diluted to 100000 cells/mL, and 100 μL of the prepared cell suspension was added to each well. After 2–3 h of incubation under standard culture conditions, the number of SKBR3 cells that settled on the SiN window was checked, and microchips containing at least 15–20 cells per window were transferred to new wells prefilled with 200 μL of DMEM for further cultivation. Prior to the experiment, cells were serum-starved overnight in FBS-free DMEM to enhance membrane expression of ErbB2. For the QD-labeling of cells, chips were rinsed once in GS-BSA-GEL-PBS (1% goat serum (GS), Rockland Immunochemicals Inc.; 1% BSA (molecular biology-grade albumin fraction V, Carl Roth GmbH-Co. KG); 0.1% cold water fish skin gelatin Sigma-Aldrich; in PBS (pH 7.4)) and then incubated in the same solution for 5 min at 37 °C to block unspecified binding of biotin-conjugated anti-ErbB2-Affibodies (ZEERB2-4772, ErbB2-AFF-B). Next, microchips were incubated for 10 min at 37 °C with 200 nM ErbB2-AFF-B in GS-BSA-GEL-PBS, and after being washed twice with 1% BSA–PBS, once in PBS, and once in cacodylate buffer (CB, 0.1 M sodium cacodylate trihydrate, Carl Roth GmbH, and 0.1 M saccharose, pH 7.4), the cells were fixed at RT with 3% formaldehyde (Electron Microscopy Sciences) in CB to prevent QD-induced clustering of ErbB2 molecules. Subsequently, cells were rinsed once with CB, three times with PBS, and incubated in 0.1 M glycine in PBS for 2 min. After two additional washes with PBS, cells were incubated in 5 nM streptavidin-conjugated Qdot 655 (Life Technologies, Carlsbad, CA, USA) in 40 μM borate buffer (sodium tetraborate boric acid, Sigma-Aldrich, pH 8.3) at RT for 12 min. Next, cells were washed three times in 1% BSA–PBS and subjected to fluorescence imaging. After that, the cells were washed once with CB and fixed for 10 min at RT with 2% glutaraldehyde (electron microscopy grade, Carl Roth GmbH-Co. KG) in order to increase stability of the samples under electron beam radiation. The combined fixation with paraformaldehyde and glutaraldehyde cross-links and immobilizes proteins in the plasma membrane. Further details on the labeling method for ErbB2 in SKBR3 cells using specific Affibodies and QDs including control experiments are described elsewhere. Also, practical details of the protocol are reported as video publication.

**Graphene Deposition.** Monolayer graphene was grown on 25 μm thick polycrystalline Cu films by CVD. The Cu foil was untreated prior to growth, leading to domain sizes of ~10 μm in lateral dimensions showing an average Raman D/G peak intensity ratio of <5%. It was covered with a polymer by spin-coating (poly(methyl methacrylate), 4 wt % in anisole, 950 K molecular weight), and the Cu catalyst was removed from the graphene by etching with (NH4)2S2O8. The as-released polymer-supported graphene was rinsed in Milli-Q water to eliminate residual etchant. It was then lifted out onto the surface of a second, slightly larger piece of Cu film that had undergone the same monolayer graphene CVD process. The stack was dried at ~50 °C to obtain a bilayer graphene film on Cu. Now the Cu was etched as before. The polymer-supported bilayer graphene was rinsed and then floated on an aqueous solution saturated with NaCl, allowing...
it to be lifted out onto the surface of a cleaved single crystal of NaCl (Structure Probe, Inc.), without significant dissolution of the substrate. The sample was then dried at ~50 °C, and the polymer was dissolved by immersion in acetone. For transfer of the graphene bilayer films onto the hydrated cells grown on microchips, the graphene–NaCl crystals were carefully placed on the surface of desalinated water. After the underlying NaCl was dissolved completely, the freely floating graphene film was lifted out with the microchip using a pair of Teflon-coated forceps (Figure S1). Afterward, the positioning of the graphene film over the SiN window was verified with a binocular and carefully corrected if necessary. Next, the microchip was fixed to a holder and left to air-dry at RT for about 5 min. The integrity of the graphene film on cells was checked by scanning electron microscopy. Only cells covered by an undamaged graphene film were selected for further investigation.

Fluorescence Microscopy. Cells grown on microchips were imaged with an inverted light microscope (Leica DMI 6000B) in darkfield (Everhart-Thornely detector) at 10.00 keV beam energy (FEI Quanta 250 FEG). The microchips were mounted on pin detection mode (Everhart-Thornely detector) at 10.00 keV beam energy (FEI Quanta 250 FEG). The microchips were imaged with a SEM in standard secondary electron overview pictures of the complete SiN window these were manually corrected if necessary. Next, the microchip was imaged with an electron beam of 200 keV. In STEM mode, images were acquired with a transmission electron microscope (JEM-ARM 200F, JEOL) equipped with a cold field emission gun and a STEM probe corrector (CEOS GmbH). The microchip was mounted on a standard single tilt TEM sample holder (JEOL), and the sample was imaged with an electron beam of 200 keV. In STEM mode, images were recorded with a pixel dwell time of τ = 20 μs. The electron probe size was 6×6 pixels (aperture CL2-3 with 20 μm diameter) with a probe current of I_p = 20 pA (spot 6×6) and beam current of 0.1 nA. The sample stage was slightly tilted (−1°), and the operational vacuum was run at 10.34 × 10⁻⁴ mbar to 1.05 × 10⁻⁴ Pa.

Scanning Transmission Electron Microscopy. STEM images were acquired with a transmission electron microscope (JEM-ARM 200F, JEOL) equipped with a cold field emission gun and a STEM probe corrector (CEOS GmbH). The microchip was mounted on a standard single tilt TEM sample holder (JEOL), and the sample was imaged with an electron beam of 200 keV. In STEM mode, images were recorded with a pixel dwell time of τ = 20 μs. The electron probe size was 6×6 pixels (aperture CL2-3 with 20 μm diameter) with a probe current of I_p = 20 pA (spot 6×6) and beam current of 0.1 nA. The sample stage was slightly tilted (−1°), and the operational vacuum was run at 10.34 × 10⁻⁴ mbar to 1.05 × 10⁻⁴ Pa.

Particle Analysis. For the detection of QD-labeled ErbB2 membrane proteins in electron microscopy pictures, our group programmed an automated procedure in ImageJ (NIH). First, a Gaussian filter with a radius of 1 pixel was applied for noise filtering. Potential variations in the image background were filtered by using a Fourier filter. Next, the image was binarized by applying an automated threshold with maximal entropy settings. Bin width was set to 5 μm, and particles with a size >10 nm were taken into account by the program. Contamination particles much larger than the QD labels were present in some images. These were grayed out manually in order to avoid the triggering of the particle detection at their corners.

To conduct the subsequent statistical analysis by pair correlation function g(r), a locally designed software tool in C++ was applied. The pair correlation function was defined as:

\[ g(r) = \frac{1}{\rho^2} \frac{1}{\pi r^2} \sum_{i=1}^{N} \sum_{j=1}^{N} k(r - |x_i - x_j|) \]

with \( r \) representing the radial distance and \( \rho \) the labeling density in the image. The covariance function \( \gamma \) and the kernel \( k \) are defined elsewhere. A Gaussian filter with a radius of 1 pixel was applied for noise filtering. The line scans were obtained with ImageJ (NIH) for a line width of 3. For each image acquired at a certain sample thickness, five randomly chosen QD labels, which were clearly identifiable as bullet-shaped particles, were selected, and a line was drawn perpendicular to the long side. The average of the background level was set to zero in the line scan at the left side of the peak. This background level was not always flat but sometimes gradually changed from the left to the right side of the line scan, due to the increasing cell thickness, which in turn increased the background signal. Therefore, the values at both sides were determined and the resulting mean value taken as described later.

Next, the data were normalized so that the peak level represented a value of 1. The resolution was determined from the standard deviation. The resolution was calculated from the 25%–75% edge width of the peaks over the full width at half maximum (FWHM). The latter angle refers to the collection area of the detector. For liquid thickness measurements, the current passing through the detector was measured. The corresponding semiangle was measured and amounted to 43 mrad. The image size was 1024 × 1024 pixels unless specified otherwise. For most recorded images, the STEM probe size was much smaller than the pixel size, and the pixel size was chosen to reduce the dose per pixel.

In TEM mode, the images were recorded at corresponding magnifications with a GIF CCD camera (Gatan) and an exposure time of 3.21 s.

Determination of the Sample Thickness. The thickness of the specimen at each imaged location was determined based on the probe current transmitted through the opening of the ADF detector. The sample thickness was calculated using the following equation for electron scattering including both the sample and the SiN membrane:

\[ t_{\text{sample}} = \exp \left( \frac{I_{\text{sample}} - I_{\text{SiN}}}{I_{\text{sample}} - I_{\text{screen}}} \right) \]

with \( I_0 \) the current density measured on the phosphor screen of the electron microscope for vacuum (no sample) and \( I_{\text{screen}} \) the current density measured with a sample inserted. The current at the phosphor screen passed through the opening of the ADF detector and measures the nonscattered fraction of the electron beam. The thickness of the SiN window was \( t_{\text{SiN}} = 50 \) nm. The total sample thickness was referred to as \( t_{\text{sample}} \). The electron mean free path length, \( t_{\text{mean}} \), measures elastic scattering into an opening semiangle \( \beta = 43 \) mrad or larger for amorphous Si₃N₄, which can be calculated using equations described elsewhere. The density for amorphous Si₃N₄ was \( \rho = 3.2 \) g/cm³, the...
atomic weight \( W = 20 \text{ g/mol} \), and the square average atomic number \( \langle Z \rangle = \sqrt{Z_{\text{Si}}^2 + Z_{\text{N}}^2} \) = 10.6, leading to a value of \( t_{\text{SiN}} = 0.89 \mu \text{m} \). Assuming the sample consisted mostly of water, and using the square average (\( Z \)) = 4.7 of water, it follows that \( t_{\text{sample}} = 4.1 \mu \text{m} \). This method is accurate within 20% compared to thickness measurements via sample tilting for micrometer-thick liquid layers. Note that the actual thickness may have differed because the density of protein is higher and the density of lipid is lower than that of water. Equation 2 can be solved to obtain the sample thickness as

\[
t_{\text{sample}} = \left( -\ln \left( \frac{t_{\text{screen}}}{l_0} \right) \right) \left( \frac{t_{\text{SiN}}}{l_0} \right) \text{sample}
\]

(3)

**Calculation of the Electron Dose during STEM Imaging.** The average electron dose \( D \) applied during STEM imaging was approximated from

\[
D = \frac{l_e r}{e d^2}
\]

per image by the division of the product of the probe current \( I \) and the pixel dwell time \( \tau \) by the product of the elementary charge \( e \) and the pixel size \( d \).

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b05258. Number of analyzed files, particles, and calculated densities; graphene deposition on cells; potential imaging artifacts; characteristics of graphene-covered and noncovered samples; additionally imaged and analyzed TNT; images of flat and ruffled cell regions; QD displacement during STEM imaging (PDF)

TNT dynamics in living SKBR3 cells (AVI)

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**Notes**

The authors declare no competing financial interest.

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