The stoichiometry of the TMEM16A ion channel determined in intact plasma membranes of COS-7 cells using liquid-phase electron microscopy

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1. Introduction

The TMEM16 or anoctamin family comprises membrane proteins with high sequence conservation but diverse functions (Pedemonte and Galietta, 2014; Whitlock and Hartzell, 2017). Whereas TMEM16A (Ano1) and TMEM16B (Ano2) of this family are calcium-activated chloride channels (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008) TMEM16C to TMEM16J may function as lipid scramblases (Brunner et al., 2014; Gyobu et al., 2015; Malvezzi et al., 2013; Suzuki et al., 2010, 2013). A homodimeric stoichiometry of the TMEM16 family of proteins has been reported by biochemical studies (Fallah et al., 2011) and fluorescence resonance energy transfer (Sheridan et al., 2011); the dimeric organization (Tien et al., 2013) has been confirmed by X-ray crystallography of a TMEM16 member of the fungus Nectria haematococca, nTMEM16 (Brunner et al., 2014). Because of the high sequence conservation (Brunner et al., 2014; Pedemonte and Galietta, 2014; Whitlock and Hartzell, 2017), the homodimeric structure of all members of the TMEM16 family can be assumed. Each single subunit comprises 10 membrane-spanning helices and its N- and C-termini are located at the cytoplasmic side of the membrane (Brunner et al., 2014). An important question is if TMEM16 resides always in a dimeric configuration in the plasma membrane or whether monomers of the protein are also present or if mixed forms exist containing clusters of dimers or larger channels exist as well. Such information is difficult to obtain with biochemical methods that rely on the extraction of protein from pooled cellular material of non-intact cells.

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2. Materials and methods

2.1. Materials

COS-7 cells (CRL-1651) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Dulbecco’s Phosphate Buffered Saline (DBPS) was from Lonza Cologne GmbH, Cologne, Germany. Dulbecco’s Modified Eagle’s Medium Glutamax™, with high glucose and pyruvate (DMEM), Fetal Bovine Serum, certified, heat inactivated (FBS), Normal Goat Serum (GS), Gibco Opti-MEM I Reduced-Serum Medium, and Quantum Dot Serum, certified, heat inactivated (FBS), Normal Goat Serum (GS), and molecular biology grade bovine serum albumin fraction V (BSA), and sodium cacodylate trihydrate were from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Electron microscopy grade gelatin, erol, 0.72 M phosphate buffered saline (PBS), 10× solution, electron microscopy grade glutaraldehyde (GA) 25% solution, D-saccharose, glycine, biotin free Technologies, Carlsbad, CA, USA. CellStripper was from Mediatech, Chantilly, VA, USA. 35 mm-cell culture dishes were from Greiner bio-one, Frickenhausen, Germany. CellStripper was from Mediatech, Chantilly, VA, USA. HPLC grade acetone and ethanol, phosphate buffered saline (PBS), 10× solution, electron microscopy grade glutaraldehyde (GA) 25% solution, D-saccharose, glycine, biotin free and molecular biology grade bovine serum albumin fraction V (BSA), and sodium cacodylate trihydate were from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Electron microscopy grade formaldehyde (FA) 16% solution was from Electron Microscopy Sciences; Harfield, PA, USA. HPLC grade deionized water, 0.01% poly-L-lysine (PLL) solution (mol wt 70,000–150,000), sodium tetraborate, boric acid, and gelatin from cold water fish skin (GEL) were from Sigma-Aldrich Chemie GmbH, Munich, Germany. FuGENE HD Transfection Reagent was from Promega, Mannheim, Germany. COS-7 Cell Avalancé™ Transfection Reagent was from EZ Biosystems, College Park, MD, USA. 35 mm-cell culture dishes with uncoated glass bottom were from Greiner bio-one, Frickenhausen, Germany. CELLview™ four compartments. Sample support microchips of dimensions 2.00 × 2.60 × 0.30 mm2 with a central silicon nitride (SiN) membrane window of dimensions of 50 × 400 μm or 150 × 400 μm, and a thickness of 50 nm were custom made by DENSolutions B.V., Delft, The Netherlands.

2.2. TMEM16A cDNA expression constructs

The full-length TMEM16A (anocamin1) cDNA (XP_011544329.1) was cloned from human placenta using oligodeoxynucleotide primers derived from expressed sequence tags EST CF593428 (forward primer) and EST CD628900 (reverse primer). The consensus sequence for initiation of translation in vertebrates GCC GCC ACC (Kozak, 1987) was introduced immediately upstream of the first AUG codon. The cDNA was cloned into the EcoRV site of the eukaryotic expression vectors pcAGGS-IRES-GFP (Philipp et al., 1998) and pcDNA3 (Invitrogen) and sequenced. The bicistronic expression vector pcAGGS-hTMEM16A-IRES-GFP was transferred into the EcoRV site of the eukaryotic expression vectors pcAGGS-IRES-GFP (Philipp et al., 1998) and pcDNA3 (Invitrogen) and sequenced. The bicistronic expression vector pcAGGS-hTMEM16A-IRES-GFP allows simultaneous expression of the TMEM16A and the GFP cDNAs. To generate a TMEM16A-mCherry fusion protein, the TMEM16A termination codon in the pcDNA3 vector was replaced by the cDNA encoding a 14-amino acid (aa) linker (RIQSTVPRADGRS) fused to the mCherry cDNA (Clontech) to yield pcDNA3-TMEM16A-mCherry. A similar construct was cloned to yield pcDNA3-TMEM16A-eGFP. As described elsewhere (Sheridan et al., 2011), the linker ensures that the mCherry (or eGFP) does not interfere with TMEM16A function. Using the Q5™ site-directed mutagenesis kit (New England Biolabs) Fse restriction sites were inserted following nucleotides 1135 to 1137 (encoding the cysteine at position 379 of TMEM16A, Fig. 1a) and nucleotides 2470 to 2472 (encoding the asparagine at position 824 of TMEM16A, Fig. 1b), respectively to yield pcDNA3-TMEM16A-mCherry-Fse 1137 and pcDNA3-TMEM16A-mCherry-Fse 2272. According to the structure determination of TMEM16 from the fungus Nectria haematococca (nTMEM16A) and because of the high conservation of the nTMEM16A-mCherry protein sequences the cysteine 379 and the asparagines 824 are predicted to reside within the extracellular linkers connecting transmembrane helices 1 and 2 (C379) and transmembrane helices 9 and 10 (N824) (Fig. 1). Previously, Yu et al. (2012) demonstrated the accessibility of a HA epitope inserted at N824 of the mouse (m)TMEM16A protein (Accession: Q8BBH3) to extracellularly applied antibody. Both proteins, the mTMEM16A protein and the nTMEM16A protein used in this study comprise 960 amino acid residues and share 92.1% sequence identity. The Fse recognition sequences were fused 5′ and 3′ to the cDNA encoding the 38-amino-acid streptavidin-binding peptide (Keefe et al., 2001) and the resulting DNA encoding grMDEKttG WRGHVGVL AGELEQRAR LEHHIQPGQRE Prpa (aa encoded by the added Fse recognition site in lower letters) was subcloned in the Fse cut constructs to yield pcDNA3-TMEM16A-SBP-379-m-Cherry (pCS-33) and pcDNA3-TMEM16A-SBP-824-m-Cherry (pCS-32). All expression constructs were sequenced on both strands and all plasmids were prepared by the EndoFree Plasmid Kit (Qiagen). Fluorescence pictures shown in Fig. 1d were acquired for COS-7 cells expressing the pcDNA3-TMEM16A-eGFP at an Axio Observer Z1 microscope (Zeiss) equipped with a 63x Plan Apochromat objective (Zeiss), a HXP 120 C lamp (Zeiss) and an Axiocam color CCD camera (Zeiss), using a GFP (excitation 470/40 nm, dichroic mirror 495 nm, emission >500 nm) filter set (AHF Analysentechnik AG, Tübingen, Germany).

2.3. Western blot

Lysates were prepared from non-transfected COS-7 cells and COS-7 cells transfected with the TMEM16A cDNAs, grown up to 80 to 90% confluence on 3.5 cm dishes. After removing the medium and washing with PBS cells were lysed in the presence of 150 μl Lämmli sample buffer (60 mM Tris HCl, pH 6.8, 4% SDS, 10% glycerol, 0.72 M β-mercaptoethanol). The proteins of the lysate were denatured at 37°C for 30 min, run on a 7.5% SDS-polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane. Blotted proteins were probed with anti-TMEM16A antibodies, rabbit polyclonal antibody (ab) 1192 generated in-house against the C-terminus of human TMEM16A and mouse monoclonal antibody C338794, generated against the N-terminus of human TMEM16A (LifeSpan BioSciences, Seattle, WA, USA). Primary antibodies were visualized by horseradish peroxidase-coupled secondary antibodies and the Western Lightning chemiluminescence reagent Plus (PerkinElmer). Original images were saved as TIFF files from LAS 3000 (Fujifilm) and further processed by CorelDRAW (Corel).

2.4. Preparation for cell settlement in dishes and on microchips

Microchips were prepared (Peckys and de Jonge, 2015; Ring et al., 2011) for cells by rinsing the microchips for 2 min in acetone,
followed by a 2 min rinse in ethanol (both solvents were of HPLC grade). The dried microchips were cleaned with Ar/O₂ plasma for 5 min and immediately placed in 0.01% PLL for 5 min at room temperature. Subsequently the microchips were rinsed in HPLC grade water twice, and kept in FBS supplemented cell medium until the cell suspension was ready (within 10–15 min). Preparation of the glass bottom cell culture dishes was the same, except that acetone and ethanol rinses were omitted.

2.5. Cell culture, cell seeding on microchips and cell culture dishes, transfection

COS-7 cells were cultured with cell media (DMEM, supplemented with 10% FBS), in a 5% CO₂ atmosphere, at 37°C. The cells were maintained according to the ATCC guidelines. Prior to transfection, the cells were harvested at confluency by rinsing the attached cell layer in DPBS, detachment with CellStripper (5 min

![Image of protein structure with labels](https://example.com/image.png)
at 37 °C), and quenching with cell media. The beforehand prepared microarrays were positioned at the bottom of a compartment of a plasma cleaned, and PLL-coated 4-compartment dish, filled with 250 µL of cell media per compartment. To each compartment 250 µL of harvested cells in suspension were added (Peckys and de Jonge, 2015; Ring et al., 2011). Usually 10–20 cells adhered on the SiN window area of a microchip. The dish was then incubated for 30–60 min in a 5% CO2 atmosphere at 37 °C until the cells had firmly adhered to the substrate. The density of the dish was in between 50 and 70% confluency. Transfection was done according to the supplier protocols, using FuGENE® HD or COS-7 Cell AvalancheTM Transfection Reagents (both reagents worked well, but the latter had a higher transfection rate). For localization in the plasma membrane and the stoichiometric determination of TMEM16A, COS-7 cells were transfected with the cDNA constructs encoding TMEM16A-mCherry, with the inserted SBP-tag at the fifth extracellular loop (position 824, pCS-33) or at the first extracellular loop (position 379, pCS-32). For control experiments COS-7 cells were transfected with the pCS-33 and pCAAGS-hTMEM16A-IRES-GFP DNAs at a ratio of 1:1 (µg/µg). The total amount of DNA used for cell transfection in a dish compartment was usually 0.75 µg, for the control experiments with two DNAs the amount of each DNA was 0.4 µg.

2.6. Labeling of SBP-tagged TMEM16A

The cells on microchips and in dishes were labeled 24–48 h after transfection. Strep-QD stock solution (1 µM) was diluted 1:20 in 40 mM borate buffer (sodium tetraborate, boric acid, pH 8.3), and then brought to a final concentration of 10 nM by dilution in PBS, pH 7.4, supplemented with 1% GS, and 1% BSA (GS-BSA-PBS). Cells were rinsed once, and then incubated for 10 min at 22 °C in PBS, pH 7.4, supplemented with 1% GS, 1% BSA, and 0.1% GEL (GS-BSA-GEL-PBS) to inhibit non-specific binding of Strep-QD. Subsequently, the cells were incubated with Strep-QD labeling solution for 12 min, at 21 °C. After rinsing 3× with PBS supplemented with 1% BSA (BSA-PBS) and once with 0.1 M cacodylate buffer, supplemented with 0.1 M saccharose, pH 7.4 (CB), the cells were fixed with 3% formaldehyde (FA) in CB for 10 min. Cells were rinsed once with CB, and 3× with PBS, followed by incubation in 0.1 M glycine in PBS, pH 7.4, (GLY-PBS) for 2 min, and 2× rinsing with BSA-PBS.

2.7. Light microscopy

The cells grown on microchips were imaged with direct interference contrast (DIC)- and fluorescence microscopy in BSA-PBS, whereby microchips with cells were placed in a new glass bottom dish. The cells were imaged with an inverted light microscope (DMi6000B, Leica, Germany), with a 40× objective, or a 63× oil immersion objective, and with filter sets for DIC, mCherry (540 – 580 nm excitation and 607–683 nm emission windows), GFP (460 – 500 nm excitation and 512–542 nm emission windows) and QD655 (340–380 nm excitation and >420 nm emission windows). The transfected cells were identified by the expression of the fluorescence marker mCherry, and GFP. QD emission was detected using short excitation wavelengths provided by the QD filter set. Note that the emission window for mCherry also captured a part of the QD fluorescence, but the distinct use of nonoverlapping excitation wavelengths for mCherry and for QDs allowed to discern the respective emission signals of the fluorescent protein against those of the QDs.

2.8. Cell fixation

Directly following light microscopy, cells that were grown and labeled on microchips were fixed with 2% glutaraldehyde (GA) in CB to increase the stability of the cellular material during the experiments, noting that unfixed cells would decompose during the transition of buffer to pure water as needed for the ESEM experiments. Therefore, the cells were rinsed once with CB, and fixed for 12 min at RT. Finally, the cells were rinsed once with CB, 3× with BSA-PBS, and kept in BSA-PBS at 4 °C until electron microscopy, usually performed within the next 1–7 days.

2.9. Loading a sample into the ESEM and preparation for STEM imaging

Labeled cells on microchips were imaged in liquid state with the dark field contrast mode using a scanning transmission electron microscopy (STEM) detector in an environmental scanning electron microscope (ESEM) (Quanta 400 FEG, FEI, USA) (Bogner et al., 2005). Therefore, the microchips were placed in a stage with a Peltier cooling element, the STEM detector was located underneath the sample, a detailed description of the setup for STEM in ESEM can be found elsewhere (Peckys et al., 2013). The sample was loaded by rinsing a microchip 4× in cooled (3 °C) HPLC grade water (to remove salts), briefly blotting the backside and positioning in the pre-cooled stage (3 °C). The sample was covered with 3 µL cooled HPLC grade water and 3 additional 3 µL water droplets were deposited close to the sample on the stage as supplemental water source, preventing drying out of the cells during the purging procedure of the specimen chamber. The purging was done by cycling the pressure in the chamber 5 times between 800 and 1500 Pa, leading to its filling with saturated water vapor. Afterwards, controlled reduction of the water film on the cells was achieved by decreasing the pressure to 740 Pa. ESEM-STEM imaging began after a few minutes, when the water layer was thin enough for high-resolution imaging.

2.10. Wet ESEM-STEM imaging

An electron beam energy of 30 kV, a spot size of 1 nm, a probe current of 600 pA, and working distances between 5.8–6.2 mm were used. Every sample was first imaged with low magnification to get an overview ESEM-STEM image covering all cells on
complete SiN membrane window area. These images were used to provide a spatial correlation to the light microscopic images. Afterwards, overview images were recorded at a higher magnification from individual, transfected cells, as detected from the fluorescence signatures of the light microscopic images. To discern individual TEMEM16A-bound QDs on the plasma membrane, the magnification was increased to 50,000–75,000× in combination with pixel-dwell times between 30 and 50 μs. Fixed cells have been shown to be stable during electron microscopy under these conditions (Hermannsdörfer et al., 2016). The standard image size was 1024 × 884 pixels, but numerous images with 2048 × 1886 pixels were also recorded. For the larger-sized images magnifications of 25,000–37,500× were used. Stage temperature was constantly kept at 3 °C, chamber pressure ranged between 720 and 740 Pa. These pressure and temperature settings guaranteed 100% relative humidity in the ESEM chamber as needed to warrant the constant coverage of the cells with a thin water layer (Peckys et al., 2013).

2.11. Correlative image analysis

Image processing was done with the Leica LAS microscope software and with ImageJ (NIH, version 1.49v). Images were usually fine-tuned in brightness and contrast, cropping was done for certain images as indicated in the figure captions. Matching light microscopic and ESEM overview images provided a practical way to visually identify individual cells, for a detailed description, please see (Peckys et al., 2016).

2.12. Particle detection

The positions of the QD labels were determined with an automated process programmed for use with ImageJ using a procedure described elsewhere (Peckys et al., 2015). Briefly, the individual particle positions were determined by applying noise filtering with a Gaussian filter of 1 pixel, deletion of background variations by applying a Fourier filter removing spatial frequencies a factor of 3 smaller and a factor of 3 higher than the expected particle diameter, binarization using an automated threshold with maximum entropy setting, and particle selection using the Analyze Particles tool detecting particles with an area within a factor of 5 of the expected area. Only a few particles (compared to hundreds automatically detected per image) were not detected by these procedures.

2.13. Statistical analysis using the pair correlation function

As explained in detail elsewhere (Peckys et al., 2015), the pair correlation function \( g(r) \) is defined as (Stoyan and Stoyan, 1996):

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

with radial distance \( r \), the labeling density in the image \( \rho \), the covariance function \( \gamma \), the kernel \( k \), and the distance between two points \( i \) and \( j \) indicated by the modulus \( |x_i - x_j| \) with the \( x \) the two-dimensional position \( (x, y) \) of a particle in the image. Software of local design in C++ was used to calculate \( g(r) \) of the particle positions an image, whereby the label positions were assumed to be planar. A histogram of \( r \) with a bin width of 2.5 nm was defined and the value of \( g(r) \) was calculated for each bin \( r \). The bandwidth was adjusted to 5 nm to obtain an optimal balance between a sharp response and the lowest fluctuation level of the obtained curves. The data of two or more images was averaged in such way that the average was computed from fractions weighted by the particle density. Values smaller than 10 nm were not allowed taking the size of the nanoparticles into account. The \( g(r) \) function is a two-dimensional model only and the labels are considered to be in a horizontal plane. Including spots into this calculation that are at a closer distance than the diameter of the label and thus not in a horizontal plane could result in incorrect determination of the \( g(r) \) curve.

2.14. Analysis of single- and double label densities

The amounts of single labels and double labels were automatically counted using software of local design in C++ programming language. Double labels were regarded as a pair with a center-to-center distance ≤35 nm. A few pairs were found at a distance <10 nm but these were not counted to remain consistent with the \( g(r) \) curve. Only 11 distances were ignored from all distances determined. Groups consisting of more than 2 labels were also contained for completeness. The pair density was also tested for simulated random distributions of particles at similar densities as used in the experiment. The amounts of labels were counted for all analyzed images and divided by the total analyzed area to obtain label densities. The results were then fitted using the nonlinear least-squares Marquardt-Levenberg algorithm implemented in Gnuplot.

3. Results

3.1. Expression of SBP-tagged hTMEM16A cDNAs

For the purpose of detecting single TMEM16A proteins within whole cells using liquid STEM, a nanoparticle needs to be attached to the protein. This was accomplished via including a SBP-tag in an extracellular loop of the membrane protein allowing the specific binding of streptavidin-coated QDs. Based on the X-ray structure of N. haematococca TMEM16 (Brunner et al., 2014) and the alignment of its amino acid sequence (nhTMEM16), with the mouse (m) and human (h) TMEM16A sequences, accessible localizations of the SBP-tag within hTMEM16A are predicted within the first extracellular loop linking transmembrane a-helix (TM) 1 and TM 2 (Fig. 1a), and within the fifth extracellular loop linking TM9 and TM10 (Fig. 1b). Although human hTMEM16A and the nhTMEM16 share considerable homology, the mammalian protein contains long insertions especially in the first and fifth extracellular loops not present in the nhTMEM16. The tag position was taken approximately in the middle of these extra loops at C379 and N824 for TM1-2 and TM9-10, respectively. An estimate of the minimal distance between the SBP-tags of a dimeric hTMEM16A is, therefore, given by the distance between the two corresponding phenylalanines (F) at position 216 of nhTMEM16 (39.74 Å) and asparagine (N) at position 585 (35.28 Å) (Fig. 1c) for the 379 insertion positions 379 and 824, respectively. Fig. 1d demonstrates that the hTMEM16A protein is present at the plasma membrane of COS-7 cells. The Western blot of the wild-type, hTMEM16A with a fluorescent label as control, and the tagged proteins indicates that all TMEM cDNA constructs used in this study are efficiently expressed in COS-7 cells (Fig. 1e).

3.2. Correlative fluorescence microscopy and ESEM STEM of QD-labeled TMEM16A

COS-7 cells transfected with the TMEM16A-SBP-824-mCherry cDNA were labeled with Strep-QDs, and first imaged with light microscopy. Fig. 2a is the overlay image of corresponding direct interference contrast (DIC)- and mCherry fluorescence images, showing a group of cells grown on the SiN membrane window
The mCherry-emitted fluorescence (represented in cyan) served to identify transfected cells, for example in the boxed area, in which the spatial distribution of the proteins spans the entire cell. The corresponding fluorescence image of the TMEM16A-bound QDs, recorded from the same group of cells, is shown in Fig. 2b. QD-emitted fluorescence was only detectable on transfected cells visible from the mCherry fluorescence, while non-transfected cells had negligibly low levels of non-specifically bound QDs. See, for example, the non-transfected cell indicated with an asterisk in Fig. 2a and b and further examples (Fig. A1a). Since the QD labels adhere extracellularly at the intact plasma membrane, it can be concluded that the QD-labeled TMEM16A was membrane bound.

To determine the labeling specificity of Strep-QD to TMEM16A, the cellular mCherry and QD fluorescence emission intensities were quantified and compared between transfected and non-transfected cells using DNA encoding hTMEM16a-mCherry (Fig. 2a, b, Figs. A1b, c). For this experiment the cells were grown in cell culture dishes with glass bottom instead of in a microchip. Representative 50 to 200 μm² large membrane regions were marked on the transfected cells, and the average emission intensities in the QD- and in the mCherry fluorescence channels were measured in these regions. Non-transfected cells were recognized from the DIC channel image and analyzed the same way. The relative intensity level of the QD signal was found to resemble the relative intensity of the mCherry signal (Fig. A2a). The average fluorescence intensities were finally computed for each group from a multiple of cell comparisons (Table 1). It followed that the QD-emission intensities were on average 52-fold higher in transfected than in non-transfected transfected cells for cells expressing hTMEM16a_SBP-AS824-mCherry. In combination with the correlation of mCherry and QD emission, these ratios demonstrate the highly specific binding of Strep-QDs to the SBP-tagged proteins.

**Fig. 2.** Light microscopy and scanning transmission electron microscopy (STEM) images of QD-labeled COS-7 cells expressing TMEM16A-SBP-E24-mcherry. (a) Overlay image of direct interference contrast (DIC) and mCherry fluorescence (displayed in cyan) showing a group of cells, grown and transfected on a SiN membrane microchip. Transfected cells are recognized by their mCherry signals. An example of a non-transfected cell is indicated with an asterisk. (b) QD-fluorescence image of the same cells shows exclusively QD signals on mCherry positive cells. (c) Electron microscopic overview image of the cell marked with yellow rectangles in a and b. Because the cells were kept under a thin water film during imaging in the ESEM chamber, all ESEM-STEM images were recorded from fully hydrated cells. (d) ESEM-STEM image recorded with 50,000× magnification from the marked area of the boxed cell in (c). The electron dense cores of individual QD labels indicate the locations of individual TMEM16A proteins. Arrows point to several examples of double-labeled protein dimers. A rare cluster of 3 labels is located within the circle. (e) The pair correlation function $g(r)$ from all labels ($n = 1541$) detected on a series of high-resolution images from this cell shows a marked peak at ~20 nm, thus revealing the dimeric state of TMEM16A.
A typical transfected cell, boxed in Fig. 2a and b, is shown in the low magnification ESEM-STEM image of Fig. 2c. Such overview images served as orientation for identifying cellular positions at the edge of the cell appropriate for high resolution ESEM-STEM. The small yellow box in this cell marks the position of the ESEM-STEM image recorded with 50,000× magnification, shown in Fig. 2d. The achieved spatial resolution of 3 nm on QD labeled proteins in the ESEM-STEM approach (Peckys et al., 2015) allows discerning the electron dense cores of individual QDs, each representing the position of an individual TMEM16A protein. However, it should be noted that the labeling efficiency is not 100%. For each transfected cell to be analyzed, several regions were imaged along the outer edge of the cell where it was thin enough for high-resolution ESEM-STEM (Fig. A3). The QD label density varied between the imaged regions, for instance from the cell shown in Fig. 2a–c a total of 17 ESEM images was on average 12.1 ± 9.3 labels/μm², with a maximum of 35.4 ± 20.5 labels/μm² and a minimum of 1.4 ± 0.9 labels/μm² reflecting the local variations of protein density in the plasma membrane. Arrows in Fig. 2d point to examples of paired proteins with a typical QD-center-to-center distances of ~20 nm. Many single labels are also present as well as a rare example of a cluster of three labels.

### 3.3. Statistical analysis of label pairs

These pairs seem to indicate the presence of TMEM16A dimers but in order to rule out random positioning into pairs, a statistical analysis of the label distances was conducted. Analysis of the pair correlation function g(r) from a total of 1541 detected particles in 17 images, covering 139 μm² of this cell, resulted in the graph shown in Fig. 2e. The value of the g(r) represents the likelihood of two labeled TMEM16A proteins occurring at a certain distance (r), whereby a value of unity represents a random distribution.

The prominent peak at 20 nm shows that this distance occurred much more often than what would be expected for a random label distribution and so an underlying biological mechanism for the formation of the pairs is likely (Peckys et al., 2015). An approximate molecular model was constructed of the TMEM16A dimer with attached QD labels (Fig. 3). The X-ray structure of *N. haematococca* TMEM16 (Brunner et al., 2014) was used for this model, noting that the SBP positions in hTMEM16A are inserted in a relatively long loop not present in *n*TMEM16 so that uncertainty exists about the exact distance between the label binding positions. This model shows that a label distance of 20 nm is a realistic value for a dimer with bound labels.

Further data was collected from another 24 cells expressing TMEM16A-SBP-824-mCherry imaged with correlative light microscopy and ESEM-STEM. The total of 25 cells yielded information from 10,731 detected particles, covering a total membrane area of 1700 μm², with an average density of 6.3 labels/μm². The pair correlation function of the pooled label position data is shown in Fig. 4 (black line). The value of the dimer peak at 20 nm is a factor 12 larger than the random level, which shows together with the absence of any other preferred distances separately visible in the curve that TMEM16A is present in dimeric form. But since many single labels are visible in the ESEM image it must be concluded that a fraction of the protein resided as monomer and/or was present as dimer but had incomplete labeling.

### 3.4. Control experiment on label-induced pairing

An important question is whether the observed label pairs are attached to actual TMEM16A dimers or whether pairs of labels are adhered to proteins regardless of their stoichiometry. A first possibility is that the Strep-QD labels pair by themselves prior to incubation with the cells. Strep-QD labels did not bind in pairs to...
monomeric binding positions in previous work for the labeling of HER2 proteins using an Affibody-biotin tag (Peckys et al., 2015). A control experiment was carried out in which Strep-QD labels were incubated with a microchip without cells but coated with a layer of immobilized biotin. ESEM-STEM images were acquired (Fig. A4), and label positions analyzed. The corresponding \( g(r) \) curve (Fig. 4) is around 1 indicating a random distribution. The label is thus not present in a pre-clustered form in significant amounts.

A second possibility is that the presence of multiple streptavidin proteins per QD induces TMEM16a dimerization. If this had happened, however, we would not have observed paired labels but rather single labels. Too rule out further possibilities and to ensure the observed \( g(r) \) curve provides information about the TMEM’s stoichiometry, we tested the influence of a changed stoichiometry. A control experiment was conducted in which the share of TMEM16s proteins having an SBP-tag was decreased by letting the cells co-express untagged TMEM16a. Therefore, the cells were transfected with a 1:1 mixture of hTMEM16A-SBP-824-mCherry and non-tagged hTMEM16A-IRES-GFP cDNAs. Transfection, labeling and imaging was done as for the aforementioned experiments, except that another fluorescence channel was added to allow the imaging of GFP fluorescence. Light microscopic examination (Fig. 5) verified that QDs specifically bound to cells expressing TMEM16A-SBP-824-mCherry (compare Fig. 5c and d). QDs did not bind to cells expressing untagged TMEM16A only (dotted white ovals). Moreover, cells expressing neither form for

\[ g(r) \]

\[ r (\text{nm}) \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

\[ 2 \]

\[ 4 \]

\[ 6 \]

\[ 8 \]

\[ 10 \]

\[ 12 \]

\[ 14 \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

\[ 2 \]

\[ 4 \]

\[ 6 \]

\[ 8 \]

\[ 10 \]

\[ 12 \]

\[ 14 \]

Fig. 4. Pair correlation functions \( g(r) \) derived from three different TMEM16A expression experiments. The function \( g(r) \) was determined from 1) the data of COS-7 cells expressing hTMEM16A-SBP-824-mCherry (black curve, 25 cells, 10,731 labels), 2) hTMEM16A-SBP-379-mCherry (red curve, 6 cells, 5291 labels), 3) a 1:1 mixture of TMEM16A-SBP-824-mCherry and non-tagged TMEM16A-IRES-GFP (blue curve, 6 cells, 2685 labels), and 4) Streptavidin-QDs randomly bound to a biotin coated SiN surface (green curve, 24 images, 4506 labels). All COS-7 graphs show marked peaks, for 1) and 3) the preferred peak distance was \( \sim 20 \text{ nm} \), and for 2) it was \( \sim 15 \text{ nm} \). The smaller peak value found in cells expressing a 1:1 mixture of untagged and tagged (at position 824) TMEM16A indicates that in these cells a large fraction of TMEM16A dimers includes one protein without tag.

Fig. 5. Light microscopy images of a control experiment showing COS-7 cells after transfection with DNA encoding both untagged hTMEM16A-IRES-GFP and TMEM16A-SBP-824-mCherry at a ratio of 1:1 (µg:µg) for QD labeling. (a) DIC image showing all cells present in the imaged area. Dotted white ovals mark cells transfected with untagged hTMEM16A-IRES-GFP only. In the upper right part of the image two cells are marked with asterisks, which do not show any GFP expression and also no mCherry, indicating that neither form of hTMEM16A cDNA is expressed. (b) Fluorescence image of the green channel reflecting cells with GFP, that is cells transfected with hTMEM16A-IRES-GFP. (c) Fluorescence image showing cells transfected with TMEM16A-SBP-824-mCherry (cyan). (d) Fluorescence image revealing cells with attached QD labels (magenta).
TMEM16A also did not bind QDs. But the unspecific background labeling in this experiment was higher than for the non-mixed TMEM16A experiment, and amounted to 1/7th of the total (Table 1).

Six cells expressing both TMEM16A constructs were examined. The pair correlation analysis was calculated from 2685 particles, covering a membrane area of 555 μm², with an average label density of 4.8/μm², and is represented in Fig. 4 as blue line. In agreement with the results from the first single expression experiment, the graph shows the same preferred distance of 20 nm as found in cells expressing the tagged TMEM16A alone. However, the peak value was reduced by a factor of 3 confirming that the relative amount of double labeled dimers was reduced for these cells that is, a fraction of dimers consisted of one tagged and one non-tagged protein. Note that the height of the g(r) peak does not scale linear with the amounts of dimers but reflects relative probabilities. If on the contrary, the labels had paired themselves, we would have obtained a similar g(r) curve for both experiment and control. The 20-nm peak in the g(r) curve thus measures relative amounts of TMEM16A dimers and can be influenced by changing the dimer concentrations.

As a second test of the labeling, we also examined cells expressing hTMEM16A-SBP-379-mCherry. This is a similar TMEM16A DNA construct, only differing with respect to the position of the SBP-tag, which is inserted at position 379, thus in the extracellular loop between TM1 and TM2. Transfection, Strep-QD labeling and imaging of TMEM16A-SBP-379-mCherry expressing cells were performed identical to the first experiment. High labeling specificity was obtained as well as a negligible unspecific background labeling (Fig. A1b, Fig. A2h, Table 1). The pair correlation analysis for TMEM16A (AS379) construct was calculated from 5291 particles, recorded from 6 cells, covering a membrane area of 360 μm², with an average label density of 14.7/μm², and is depicted in Fig. 4 as a red line. Similar as for the graph of other TMEM16A protein, one peak rises above the random level, although here it is at the distance of 15 nm. The position of the g(r) peak was thus influenced by the tag position. On account of the uncertainty of the structure of the inserted loop for hTMEM16A it is impossible to relate the observed difference in the label difference with structural properties but this experiment serves as control experiment verifying that g(r) measures by the protein stoichiometry.

3.5. Analysis of densities of single labels and label pairs

The densities (labels/analyzed membrane area) of observed single labels N1 and labels in pairs N2 (see Table 2) were analyzed in order to estimate the labeling efficiency η, and to be able to conclude on the presence of TMEM dimers versus monomers. Densities of pairs were measured from the amounts of labels positioned at a distance of maximal d = 35 nm. This distance enclosed the width of the main peak of the g(r) curve (Fig. 4) reflecting dimers. Larger clusters than 2 labels were also counted. A total of 11% of the labels was found in pairs for the TMEM16A-824. But this number should be considered with caution. In addition to real dimers, the measured number of pairs may also be influenced by the presence of i) monomers positioned at a close distance by random chance, ii) two dimers or a monomer and a dimer at close distance with each having only one label attached, iii) larger protein complexes than dimers with incomplete labeling, and iv) QD labels pre-clustered in pairs attached to any protein. The density of randomly positioned labels within 35 nm distance rd was determined from a simulated image of randomly positioned labels at the same density as the experiment (Table 2), and can thus be corrected for. Higher-order multimers are not likely present because other methods reported a homodimeric stoichiometry of the TMEM16 family of proteins (Brunner et al., 2014; Fallah et al., 2011; Tien et al., 2013).

The control experiment using a sample with randomly distributed strep-QD labels bound to a surface coated with biotin, revealed that the distribution of detected single labels and pairs was similar (within ±0.02/μm² deviation) to a simulated random distribution (Table 2). Combined with the absence of a peak in the g(r) function (Fig. 4), it can thus be concluded that pre-formed labels did not exist within the accuracy of our measurement. The analysis of densities of N1 and N2 also revealed a small fraction (2% of the total number of labels for TMEM16A-824) in clusters of 3 or more labels (Table 2). This fraction presumably contained multiple dimers (or monomers) randomly placed at close distance, or possibly clustered by association in lipid rafts (Jin et al., 2013; Sones et al., 2010). Indeed, the value of g(r) is larger than 1 (Fig. 4) also beyond the main peak indicating some level of clustering of the proteins. This fraction was added to N2 in the further analysis to count all paired TMEM.

In order to determine the density of dimers, it was assumed that each dimer had two independent binding sites with equal η. The probability to observe a dimer labeled with two labels thus equals η² · m2, with the density of actual dimers given by m2 (N2 = 2 m2 for 100% labeling efficiency). The value of md needs to be added in a quadratic manner to the density of dimers as both numbers are uncorrelated. The probability of finding a dimer with one attached label only is 2(1-η)η · m2, which presumably contained multiple dimers (or monomers) randomly placed at close distance, or possibly clustered by association in lipid rafts (Jin et al., 2013; Sones et al., 2010). Indeed, the value of g(r) is larger than 1 (Fig. 4) also beyond the main peak indicating some level of clustering of the proteins. This fraction was added to N2 in the further analysis to count all paired TMEM.

The presence of unspecific labels was neglected since it represented only a fraction of 1/42 of the number of labels (Table 1). This set of equations cannot be solved without knowing η. Additional data was, therefore, used in which the TMEM16A had its SBP tag at position 379. This data had different values of m1, m2, m3, m4, md, fitting Eqs. (2) and (3). The respective total amounts of labels N and N0 equal:

\[ N = \eta \cdot (m_1 + 2m_2) \]  
\[ N_0 = \eta_b \cdot (m_{1b} + 2m_{2b}) \]  

The ratio N/N0 = 0.42 is known from the experiment (Table 1). The ratio of η and ηb was determined from analyzing the fluorescence data (Table 1). Although the fluorescence measures were

### Table 2

<table>
<thead>
<tr>
<th>Tag</th>
<th>N</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5.47</td>
<td>0.72</td>
<td>0.13</td>
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<td></td>
<td></td>
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<td>0</td>
</tr>
<tr>
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<td>0.83</td>
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<td></td>
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<td>0.73</td>
<td>0.04</td>
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<td>4.35</td>
<td>0.35</td>
<td>0.09</td>
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<td>rnd</td>
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<tr>
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<td>16.39</td>
<td>0.95</td>
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<td>rnd</td>
<td></td>
<td>16.41</td>
<td>0.94</td>
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</tr>
</tbody>
</table>
not calibrated in absolute levels, their relative values can be compared. The quotient of the QD- and the mCherry fluorescence is a measure of the relative labeling efficiency. Comparing these values between both mutants provided the ratio \( \eta / \eta_p = 0.8 \). Substituting this information into Eqs. (4) and (5) eliminated \( n \) as well as one density parameter; we eliminated \( m_1 \) via:

\[
m_1 = \frac{N_{q_b}}{N_{q_s} q_b^2} \cdot (m_{1,b} + 2m_{2,b}) - 2m_2
\]

(6)

It was thus possible to determine a unique combination of parameters fitting the experimental data via Eqs. (2) and (3). To increase the accuracy of the fitting, we also included data of the experiment using the mixed DNA in which TMEM16A with SBP tag was mixed with TMEM16A without tag. Here, the density of TMEM16A-SBP/TMEM16A(no tag) = \( s \). The average densities of \( m_1 \) and \( m_2 \) as well as \( \eta \) were assumed to be equal in this experiment compared to the experiment in which all TMEM16A contained a SBP tag. A complicating factor was that fluorescence data showed that the mixed data exhibited a non-negligible density of unspecific labels visible as spots in Fig. 5. Therefore, a background level \( bkg = 0.7 \) (Table 1) was added. The probability of finding labels attached thus follows as:

\[
N_{1,s} = \eta \cdot \left( s \cdot m_1 + 2(1 - s) \cdot m_2 \right) + 2(1 - \eta) \eta_s^2 \cdot m_2 + bkg
\]

(7)

\[
N_{2,s} = \sqrt{\left(2\eta_s^2 \cdot m_2\right)^2 + \text{Rnd}_{\text{max}}^2}
\]

(8)

The factor \( s \) was unknown even though the mixed experiment was carried out in equal DNA amounts because the exact protein expression may differ somewhat, and the cells selected for the experiment might not have an exact ratio of 50%. Now, with two more measurements \( (N_{1,s} \text{ and } N_{2,s}) \) and only one further unknown parameter, it was possible to determine all parameters and their error margin. We obtained the values \( \eta = 0.17 \pm 0.01, m_2 = 15 \pm 3/\mu m^2, m_{1,b} = 0 \pm 8/\mu m^2, m_{2,b} = 35 \pm 8/\mu m^2, \) and \( s = 0.8 \pm 0.1 \). It thus follows that \( \eta_0 = 0.21 \pm 0.01 \). The labeling efficiency for the TMEM is similar as obtained by others in experiments using gold nanoparticle labels (Fiala et al., 2013). Substituting these values back into Eq. (6) results in \( m_1 = 8 \pm 6/\mu m^2 \). The densities of labels in pairs of 30 and 70/\mu m^2 for TMEM16A-A284 and TMEM16A-A379, respectively, were much larger than the densities of monomers. Moreover, the determined densities of monomers are within the error margin of this fit. It is thus concluded that TMEM16A is present mostly as homodimer in the plasma membrane, whereas the data did not reveal significant fractions of TMEM16A residing as monomer in the plasma membrane.

4. Discussion

By combining a labeling approach consisting of a SBP tag linked to a streptavidin-conjugated nanoparticle, and subsequent imaging with correlative light microscopy and liquid-phase electron microscopy, we determined the stoichiometry of the TMEM16A protein in the intact plasma membrane of COS-7 cells. For this purpose, the inter-label distances of QD label positions were statistically analyzed including the control experiments. High-resolution ESEM-STEM images revealed the presence of single labels and pairs of labels with center-to-center label distances smaller than 35 nm. The pair correlation function showed clearly preferred distances (peak in the \( g(r) \) curve) of 20 nm, respectively 15 nm (note that the bin width was 2.5 nm) for the protein variant with a different SBP-tag position, between the centers of two labels and the range of these distances is consistent with a schematic model of QD labels bound to a TMEM16A dimer (Fig. 3). We conclude that hTMEM16A resides as dimer only and that monomers are absent by combining data of three experiments with varying protein densities.

The TMEM16A channel examined in our study is supposed to be a permanent or obligate oligomer, that is the protein occurs only in the oligomeric state, as is the case for most homodimeric proteins (Jones and Thornton, 1996). With very few exceptions, ion channels have an oligomeric arrangement (compare Brookhaven Protein Data Bank) whereby the channel consists of oligomeric complex of 2, like TMEM16 (Brunner et al., 2014), or 3, 4, 5 or 6 similar subunits with an aqueous pore at its center. It was thus expected prior to our experiment that the protein resided in dimeric form. The identical homodimeric assembly of both the TMEM16A resident in the endoplasmic reticulum and plasma membrane-bound has been reported using extracted protein complexes (Fallah et al., 2011). These authors conclude that the TMEM16A channel stably assembles as the subunits fold into a homodimeric assembly. Our results now provide evidence for data obtained from imaging proteins in the intact plasma membrane that the homodimeric form is the only form present. In contrast, from studies using single-molecule fluorescence photo bleaching experiments such as described in (Sheridan et al., 2011; Tien et al., 2013), the presence of monomers cannot be ruled out, as is done here.

The detection of proteins in intact cells is a favorable condition for direct probing of any spatial heterogeneity of biomolecules under physiological conditions, and to selectively examine specific local substructures of interest within the plasma membrane. So far, the only strategy for the examination of protein stoichiometry in the natural environment of the plasma membrane was subunit counting by single-molecule fluorescence photobleaching (Hines, 2013; Ulbrich and Isacoff, 2007). This technique determines the stepwise reduction of the fluorescence intensity to count the number of fluorescent protein-tagged subunits in the membrane protein complex of interest. It is an indirect technique requiring a very low density of the expressed proteins, usually obtained in Xenopus laevis oocytes, in the range of 1–2 proteins/\mu m^2, and strict surveillance of several other demanding technical efforts to prevent over counting (Shivanandan et al., 2014; Veatch et al., 2012). The challenges associated with the photobleaching techniques might lead to differences in reported subunit numbers, as is the case, for instance, for the calcium release-activated calcium (CRAC) channel ORAI (Demuro et al., 2011; Hou et al., 2012). Electron microscopy is typically used for thin sections but several approaches are available to image whole cells in liquid (de Jonge et al., 2009; Maruyama et al., 2012; Nishiyama et al., 2010; Peckys et al., 2013) or in an ice layer (Elbaum et al., 2016; Kourkoutis et al., 2012) allowing nanometer resolution as required to examine protein complex stoichiometry.

A particularly important advantage of ESEM-STEM detection is that labeled proteins can be studied at endogenous protein concentrations for example, EGFR on COS7 cells (Peckys et al., 2013), as well as for overexpressed concentrations, for instance of HER2 in breast cancer cells (Peckys et al., 2015) contrasting the capabilities of super resolution fluorescence microscopy. The latter method does not have sufficient spatial resolution to resolve the individual subunits of membrane protein complexes, while indirect methods such as Förster resonance energy transfer are prone to artifacts and are incapable of handling the often high endogenous protein concentrations (Piston and Kremers, 2007).

The examination of the TMEM16A’s stoichiometry was enabled by the use of a streptavidin binding peptide tag accessible from the extracellular site of the plasma membrane to a nanoparticle. The small tag comprises the \( \sim 4.3 \) kDa-sized SBP (Barrette-Hig et al., 2013), a streptavidin of \( \sim 5 \) nm diameter, and a QD label of dimensions \( 6 \times 14 \) nm\(^2\) (Peckys et al., 2015). The resulting label-target distance of \( <10 \) nm, allows an analysis of the pair correlation function \( g(r) \) for analyzing protein complex formation and
clustering of labeled membrane proteins (Peckys et al., 2013). The SBP-tag was, similar to many other peptide tags, originally designed to facilitate the purification and detection of recombinant proteins (Keefe et al., 2001). Using such tags as tools to label and study proteins through microscopy (Mc Cann et al., 2005) is another, but less frequent application. Due to their sixfold smaller size than fluorescent proteins, peptide tags reduce the risk of perturbation of protein trafficking, function, and impaired protein–protein interaction (Lotze et al., 2016). The label containing streptavidin-coated QD nanoparticles is also much smaller than conventional immunogold labels, which consist of antibody-based linkers resulting in distances of ≥30 nm between the nanoparticle label and its target (Bergersen et al., 2008), thus preventing a stoichiometric analysis.

For the purpose of stoichiometry investigation several requirements have to be fulfilled. i) A 1:1 ratio between label and protein should be provided. This condition was met by using one tag in each single TMEM16A protein, and by avoiding steric hindrance (see Fig. 3). Since the two SBP-tag binding epitopes were at opposing sides of the streptavidin molecule (Barrette-Ng et al., 2013), only one label bound to the TMEM16A protein, and a configuration in which two QD labels bind to a TMEM16A monomer was highly unlikely. ii) The labels should have access to all tags present in a protein complex, thus the tag positions and the size of the label should not restrict the binding of all labels. Dimeric label positions were indeed observed for both tagged variants of the TMEM16A protein, with tags either at position 824 or 379. Both tag positions thus allowed specific QD labeling of the TMEM16A dimer complex, as was revealed by the presence of the respective g(r) peaks. The difference in peak position and peak height between these two variants reflects differences in accessibility of the label to the different epitope landscape at and around the respective tag positions, and indicates a restriction in the spatial orientation between the respective tag in a TMEM16A protein and a bound QD.

The described method also functions for much higher membrane protein densities (Peckys et al., 2015) as a consequence of the high spatial resolution. It would still be possible to detect closely packed proteins with a pitch of d = 20 nm, and a protein density on the order of ~10⁷ μm⁻². The accuracy of the method reduces for lower densities due to unspecific labeling, which was now ~2% for TMEM-824. But allowing an accuracy in the measurement of the number of single labels of 20% that would still be acceptable for fitting the variables, a factor of 10 lower protein density would still be acceptable, and thus a density of ~0.5 μm⁻². The detection of protein complexes of higher orders than dimers is possible as we have already demonstrated for hexameric ORAI 1 proteins using a different QD label (Peckys et al., 2016) but a higher labeling efficiency would be desirable for precise analysis. The analysis of intracellular structures and protein distributions is possible but requires different protocols (Maruyama et al., 2012) since the cells are not sectioned.

For studies in which the inclusion of a SBP-tag is impossible or undesired several other labeling strategies exist based on the streptavidin-conjugated QD as nanoparticle label. A biotin-conjugated ligand of a receptor can be employed as tag, for instance, epidermal growth factor (EGF) (Peckys and de Jonge, 2015; Peckys et al., 2013). Another option is the usage of high affinity peptides, such as Affibodies (Peckys et al., 2015), and furthermore the hemagglutinin (HA)-tag binding an antibody fragment against the tag (Anti-HA Fab) has been demonstrated in combination with STEM detection (Peckys et al., 2016). These labeling methodologies differ from the here presented new approach by their need for two labeling steps, the first being the binding of the biotin-conjugated peptide, ligand or Fab to the target protein, followed by fixation of the cells, and subsequent incubation and binding of the QD labels.

5. Conclusions

We have demonstrated the applicability of a new strategy for determination and visualization of the stoichiometry of a plasma membrane resident ion channel by measuring the inter-label distances of recombinant SBP-tagged TMEM16A proteins labeled with QDs and imaged with liquid-phase electron microscopy in intact cells. Statistical analysis using the pair correlation function g(r) confirmed a dimeric conformation of the protein. The analysis of detected amounts of label pairs and single labels revealed that hTMEM16A is not present as monomer. The obtained information supplements data obtained using biochemical methods for which proteins were extracted from cells contrasting our approach in which the proteins are examined in their native state of the intact plasma membrane. We expect this new methodology to help in the elucidation of stoichiometry of membrane protein species for which this information is not yet available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2017.05.009.

References