Journal of Structural Biology 199 (2017) 1–11

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

A novel immuno-gold labeling protocol for nanobody-based detection of HER2 in breast cancer cells using immuno-electron microscopy

M. Kijanka [a,1,2], E.G. van Donselaar [b,1], W.H. Müller [c], B. Dorresteijn [a,3], D. Popov-Čeleketić [a], M. el Khattabi [d], C.T. Verrips [d], P.M.P. van Bergen en Henegouwen [a,*], J.A. Post [a]

[a] Division of Cell Biology, Department of Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[b] Department of Cell Biology, Center for Molecular Medicine, University Medical Center Utrecht, Utrecht, The Netherlands
[c] Cryo-EM, Department of Chemistry, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[d] QVQ Holding BV, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 3 March 2017
Received in revised form 23 May 2017
Accepted 24 May 2017
Available online 25 May 2017

Keywords:
Nanobody
VHH
HER2
Light microscopy
Electron microscopy
SEM
TEM

ABSTRACT

Immuno-electron microscopy is commonly performed with the use of antibodies. In the last decade the antibody fragment indicated as nanobody (VHH or single domain antibody) has found its way to different applications previously done with conventional antibodies. Nanobodies can be selected to bind with high affinity and specificity to different antigens. They are small (molecular weight ca. 15 kDa) and are usually easy to produce in microorganisms. Here we have evaluated the feasibility of a nanobody binding to HER2 for application in immuno-electron microscopy. To obtain highest labeling efficiency combined with optimal specificity, different labeling conditions were analysed, which included nanobody concentration, fixation and blocking conditions. The obtained optimal protocol was applied for post-embedment labeling of Tokuyasu cryosections and for pre-embedment labeling of HER2 for fluorescence microscopy and both transmission and scanning electron microscopy. We show that formaldehyde fixation after incubation with the anti-HER2 nanobody, improves labeling intensity. Among all tested blocking agents the best results were obtained with a mixture of cold water fish gelatine and acetylated bovine serum albumin, which prevented a-specific interactions causing background labeling while preserving specific interactions at the same time. In conclusion, we have developed a nanobody-based protocol for immuno-gold labeling of HER2 for Tokuyasu cryosections in TEM as well as for pre-embedment gold labeling of cells for both TEM and SEM.

1. Introduction

Immuno-electron microscopy combines the localization of a defined protein with fine structural details of the cell or tissue. Application of gold labeling has been demonstrated by pre-embedment and post-embedment protocols and applied both for transmission and scanning electron microscopy. Gold particles are the most often used electron dense markers, as they can be prepared in different sizes and bound to specific linker molecules such as antibodies enabling direct labeling of different proteins of interest (Faulk and Taylor, 1971; Baschong and Stierhof, 1998). Labeling efficiency strongly depends on the fixation and accessibility of the epitope. Moreover, the accuracy of gold labeling depends on the size of the linker molecule, which is in the case of antibodies not better than 10 nm (Xiaoying, 2015). Size reduction of the linker molecule might improve labeling efficiency and accuracy of gold labeling. Here we investigated the possible application of a novel type of antibody fragment indicated as nanobody, in immuno-light and electron microscopy.

Nanobodies are defined as the variable domains of the heavy chain of heavy chain-only antibodies that are found in the members of the *Camelidae* family (Hassanzadeh-Ghassabeh et al., 2013; Muyldermans et al., 2009). They are also referred to as VHHs or single domain antibodies (sdAbs). Nanobodies can be selected from immune libraries using phage display to bind to a wide variety of different proteins (Muyldermans, 2013). They have a molecular weight of 15 kDa, a size of 3 × 3 × 4 nm and can often easily be produced in microorganisms such as *E. coli* or yeast cells. Nanobodies contain three complementarity determining regions

* Corresponding author at: Cell Biology, Dept. Biology, Science Faculty, Utrecht University, Padualaan 8, 3584CH Utrecht, The Netherlands.
E-mail address: p.vanbergen@uu.nl (P.M.P. van Bergen en Henegouwen).
[1] Equal contributions.
[2] Present address: Apo-T BV, Oss, The Netherlands.
[3] Present address: Danone Nutricia Research, Utrecht, The Netherlands.

http://dx.doi.org/10.1016/j.jsb.2017.05.008
1047-8477/

(CDRs) or hypervariable loops that are interspaced by conserved framework regions (Muyldermans, 2013). In contrast to the six CDRs of the conventional antibodies, nanobodies have only three CDRs that participate in antigen recognition. Despite the smaller surface area of the paratope, nanobodies can bind with a similar affinity and specificity to their target molecule as antibodies (Muyldermans, 2013). Some nanobodies have an extended CDR3, which enables these nanobodies to enter cavities present on the surface of the antigen causing them to bind conformation-dependent epitopes (Muyldermans et al., 2009; Muyldermans, 2013). The small size might also enable the nanobody to penetrate better into the permeabilized cell or into the (cryo)section, thereby contributing to a higher immuno-labeling efficiency. Similar effects of section penetration contributing to higher labeling efficiency were previously described for differently sized gold particles (van Bergen en Henegouwen, 1986; Stierhof et al., 1986). Moreover, as a result of their small size, the accuracy of immuno-gold labeling might be improved. This small size of nanobodies, in combination with their versatility, makes them in principle attractive linker molecules for immuno-gold labeling.

We have previously selected several HER2targeted nanobodies from an immune MCF7 library (Kijanka et al., 2013). In the current study we tested the feasibility of a recently described HER2-targeted nanobody, 11A4, for immuno-EM applications on HER2 positive human breast cancer cells. HER2 belongs to the ErbB family of receptor tyrosine kinases and is known to play an important role in breast cancer (Yarden, 2001). To obtain an optimized protocol for HER2 gold labeling using nanobodies, we set out to optimize the parameters known to affect both specificity and efficiency of immuno-gold labeling. The validity of the obtained protocol was demonstrated for post-embedment, on-section labeling of Tokuyasu cryosections. Furthermore, we show the feasibility of this nanobody labeling protocol to detect HER2 by pre-embedment labeling using SKBR3 cells for both transmission (TEM) and scanning electron microscopy (SEM). Our data demonstrate that the nanobody is a highly promising novel linker molecule for applications in immuno-electron microscopy.

2. Materials & methods

2.1. Cell lines and culture conditions

Human HER2-positive breast cancer cells, SKBR3 (HTB-30), and HER2-negative breast cancer cells, MDA-MB-231 (CRM-HTB-26), were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in DMEM (Gibco) supplemented with 7.5% (v/v) FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Nanobody production

HER2 targeted nanobody, tagless 11A4 or 11A4 provided with a His- FLAG- or EPEA-tag was produced as described before and provided by QVQ B.V. (Kijanka et al., 2013). Production of nanobodies was induced by addition of 1 mM IPTG when bacteria reached log-phase. HER2-specific nanobodies were purified from the periplasmic fraction by protein-A affinity chromatography using a HiTrap protein A HP column (GE Healthcare, Zeist, The Netherlands) on the ÄKTAxpress system (GE Healthcare, Zeist, The Netherlands).

2.3. Binding study of anti-HER2 nanobody 11A4 to SKBR3 and MDA-MB-231 cells

SKBR3 or MDA-MB-231 cells were seeded at the density of 20,000 cells/well two days before the assay. Cells were fixed with 4% (w/v) formaldehyde (FA) in PHEM buffer (30 mM PIPES [Merck], 12.5 mM HEPES [Merck], 5 mM EGTA [Sigma-Aldrich], 1 mM $MgCl_2$ [Merck] pH 6.9) for 30 min at RT either before or after 1.5 h incubation with a serial twofold dilution of 11A4 nanobody (500 nM–0.19 nM range) or monoclonal antibody (mAb), trastuzumab (10 nM–0.009 nM) at 4 °C. Fixation was stopped by an 10 min incubation with 100 mM glycine in PBS. Cells were blocked with 2% (w/v) milk powder in PBS (MPBS) for 30 min at RT, followed by 1 h incubation at RT with rabbit anti-VHH protein G purified serum 976 (QVQ) in 2% MPBS and 1 h incubation at RT with goat anti-rabbit IgG conjugated to IRDye800cw. Upon 2 consecutive washing steps with PBS, the fluorescent signal was detected with an Odyssey scanner.

2.4. Preparation of Tokuyasu samples

Preparation of Tokuyasu sections from SKBR3 and MDA-MB-231 cells was done essentially as previously described (Slot and Geuze, 2007; Tokuyasu, 1973). Cells were grown in culture dishes to 80–90% confluency and then fixed by adding to the dish a equal volume of 4% (w/v) FA in 0.1 M PHEM buffer pH 6.9 (final concentration 2% (w/v)). After 15 min the fixative was replaced by fresh 4% (w/v) FA in PHEM buffer (30 mM Pipes; 12.5 mM Hepes; 5 mM EGTA; 1 mM $MgCl_2$, pH 6.9, (Sobue et al., 1988). After 2 h at rt fixation was continued overnight at 4 °C. After washing the cells five times with PHEM buffer, the cells were scraped in 1.5 ml PHEM buffer pH 6.9 containing 1% (w/v) gelatin (Gelatine 250 LP30; I.P.D., Nijmegen, The Netherlands), transferred to 1.5 ml microcentrifuge tubes (Bioplastics) and spun down at 37 °C for 3 min, 1200 rpm. After removing the supernatant the pellet was resuspended in warm 12% (w/v) gelatin in PHEM buffer and left for 5 min in an incubator (type B15, Hereaus) at 37 °C, while gently mixing two times on a vortex during this incubation. Next the cells were centrifuged for 3 min, 3000 rpm at 37 °C after which the supernatant was removed. The tube with cell pellet was kept for a few minutes in the incubator after which the remaining gelatin could be removed, leaving a small volume on top of the pellet. Cells were resuspended in this small volume to have a lower density of cells in the pellet and kept at RT to let the gelatin solidify on ice. After 15 min warm (37 °C) fresh 12% (w/v) gelatin in PHEM buffer pH 6.9 was added and allowed to solidify. After gelation, blocks of ~1 mm^3 were prepared on an ice-cooled metal plate under a stereo-microscope. The blocks were transferred to 2.3 M sucrose (Merck) in PHEM buffer pH 6.9 and placed on a rotator at 4 °C. After overnight infiltration the blocks were mounted on specimen holders and plunge frozen in liquid nitrogen.

2.5. Sectioning of Tokuyasu samples for immuno-fluorescence labeling

Following trimming of the samples on a glass knife at −100 °C with a cryo-ultramicrotome (UC6/FC6; Leica Microsystems) to a suitable block shape, 500 nm cryosections were cut on a glass knife at the same temperature. Flat, glossy-looking sections were shifted from the knife edge with a guinee pig hair mounted on a wooden stick and picked up with a wire loop and a droplet of a 1:1 mixture of 2% (w/v) methylcellulose (Sigma) in Milli-Q water and 2.3 M sucrose in 0.1 M phosphate buffer pH 7.4. Upon thawing, the sections were mounted in a wax-marked area on a silane-coated glass slide, covered with 2.3 M sucrose in 0.1 M phosphate buffer and stored at 4 °C until immuno-fluorescence labeling.

2.6. Sectioning of Tokuyasu samples for immuno-gold labeling

Following trimming of the samples on a glass knife at −100 °C with a cryo-ultramicrotome (UC6/FC6; Leica Microsystems) to a suitable block shape, 80 nm cryosections were cut on a dry

diamond knife (Element Six B.V.) at the same temperature and under control of an ionizer (Static line; Leica Microsystems). Flat ribbons of glossy-looking sections were shifted from the knife edge with a guinee pig hair mounted on a wooden stick and picked up with a wire loop and a droplet of a 1:1 mixture of 2% (w/v) methylcellulose (Sigma) in Milli-Q water and 2.3 M sucrose in 0.1 M phosphate buffer pH 7.4. Upon thawing, the sections were mounted on formvar (Fluka)-coated, carbon-stabilized copper grids (100 mesh hexagonal copper grids, Stork-Veco B.V.) and stored at 4 °C until immuno-gold labeling.

2.7. Immuno-fluorescence labeling on Tokuyasu cryosections

Upon preparation, cryosections of SKBR3 and MDA-MB-231 cells (500 nm thick) were covered with a layer of 2.3 M sucrose in 0.1 M phosphate buffer pH 7.4 and stored at 4 °C until further use. Before labeling, sucrose was removed in subsequent washing steps of five times 5 min with PBS at 37 °C and five times 3 min with PBS at RT. The sections were next incubated for 5 min at RT with sodium borohydride (1 mg/ml) in PBS followed by five washes of 2 min with PBS and 2 washes of 3 min with 20 mM glycine in PBS and blocked twice for 5 min at RT with blocking solution (either 0.225% (w/v) CFG + 0.1% (w/v) BSA-c or 10% (w/v) BSA in PBS). Sections were incubated with indicated concentrations of 11A4 nanobody in blocking solution for 1 h at RT, washed five times 2 min on droplets of 10x diluted blocking solution followed by an incubation with first rabbit anti-VHH purified serum (R1219, 20 μg/ml, 1 h, RT) followed by an incubation with goat anti-rabbit-IgG-Alexa488 (2 μg/ml, 1 h, RT), both diluted in blocking solution. After five washes of 3 min with PBS, sections were fixed with 4% FA (w/v) + 0.2% ((w/v) GA in 0.1 M phosphate buffer pH 7.4, washed ten times for 1 min on droplets of distilled water and stained with DAPI (4 μg/ml, 5 min) before embedment in Prolong Gold.

2.8. Immuno-gold (IG) labeling of Tokuyasu cryosections

Grids containing sections of SKBR3 cells or MDA-MB-231 cells were placed on PBS at 37 °C for 60 min to let the pick-up droplet diffuse away and melt the solidified gelatin present between the cells. The following steps were carried out at RT. Grids with sections were washed five times 2 min on droplets of PBS containing 20 mM glycine in PBS (Merck) and blocked for 15 min on droplets of PBS containing 1% (w/v) BSA (Bovine Serum Albumin Fraction V, Sigma-Aldrich) or 0.225% (v/v) CFG + 0.1% (v/v) BSA-c (CFG: gelatin from cold water fish skin 45% in water (Sigma-Aldrich); BSA-c: acetylated Bovine Serum Albumin 10% in water (Aurion)) to prevent a-specific binding. After blocking the sections, they were incubated for 60 min on droplets of 5 μl with indicated concentration of 11A4 nanobody in blocking solution followed by 5 washes of 2 min on droplets of 10× diluted blocking solution. Next, the grids were incubated for 60 min on droplets of 5 μl rabbit anti-llama antibody (RαVHH, 1 mg/ml, Rabbit 1216 QVQ, Utrecht, The Netherlands) 1:50 diluted in blocking solution. After six washes of 2 min on droplets of 10x diluted blocking solution the grids with sections were incubated for 20 min on droplets of 7 μl of Proteïn A coupled to 15 nm Gold (PAG15; CMC, Utrecht, The Netherlands), 1:60 diluted in blocking solution, washed seven times 2 min on droplets of PBS and fixed for 5 min with 1% (w/v) glutaraldehyde (Taab) in PBS. After 10 washes of 1 min on droplets of in Milli-Q water, the sections were then poststained for 5 min with 2% (w/v) Uranyl Acetate (EMS) in 0.15 M Oxalic Acid (Merck) pH 7.4. Subsequently, after a quick wash on two droplets of in Milli-Q water, the grids with sections were transferred to droplets of embedding solution containing 1.8% (w/v) methyl cellulose (Sigma) and 0.4% (w/v) uranyl acetate (EMS) in distilled water on parafilm on an ice-cooled metal plate. After 5–10 min of incubation, the grids with sections were picked up in a wire loop. Most of the excess of the viscous embedding solution was drained away with filter paper after which the grids with sections were air-dried forming a thin layer of embedding solution. The sections were viewed in a JEM 1010 (Jeol) at 80 kV.

2.9. Transmission electron microscopy of pre-embedment immunogold labeled SKBR3 cells

2.9.1. Labeling

SKBR3 cells cultured for three days in a dish with pieces of carbon-coated aclar (Aclar Embedding Film 2 mm thickness, EMS) were fixed by adding an equal volume of 4% (w/v) FA in 0.1 M PHEM buffer pH 6.9 (final concentration is 2% FA). After 15 min of fixation at RT the fixative/medium mixture was replaced by fresh 4% (w/v) FA in 0.1 M PHEM buffer pH 6.9. The cells were fixed for 2 h at RT followed by 16 h at 4 °C. After washing six times 5 min with PBS pH 7.4 (137 mM NaCl; 2.7 mM KCl; 8.1 mM $Na_2HPO_4 \times 2H_20$; 1,7 mM NaH_2PO_4 x H_20), free aldehyde groups were quenched with 100 mM NH_4Cl in PBS for 10 min, after which the SKBR3 cells were washed twice in PBS for 5 min and exposed for 15 min to the blocking solution containing 0.225% (v/v) gelatin from cold water fish skin 45% in water (CFG) + 0.1% (v/v) acetylated Bovine Serum Albumin (BSA-c, 10% in water, Aurion) to prevent a-specific labeling. To immuno-gold label the HER2 receptors on the surface of the SKBR3 cells, the cells were incubated with the HER2 targeted nanobody 11A4 (VHH-11A4, 3 μg/ml, QVQ, Utrecht, The Netherlands) diluted in blocking solution (0.225% (v/v) CFG + 0.1% (v/v) BSA-c/PBS)) for 1 h at RT. After three times 10 min washing with blocking solution, rabbit anti-llama antibody (RαVHH, 1 mg/ml, Rabbit 1216, QVQ, Utrecht, the Netherlands) 1:50 in 0.225% (v/v) CGF + 0.1% (v/v) BSA-c/PBS was used as bridging antibody for 1 h at RT. Thereafter the cells were washed three times 10 min with blocking solution, and subsequently incubated with protein-A gold 15 nm (PAG15, CMC, Utrecht, the Netherlands) 1:60 diluted in blocking solution for 20 min at RT to mark the antigen-antibody complex. Thereafter the cells were thoroughly washed for three times 10 min with PBS and three times 5 min with PBS.

2.9.2. Positive control

As a positive control, SKBR3 cells were blocked with 1% (w/v) BSA (Albumin, Bovine fraction V) in PBS, then HER2 receptors present on the surface of SKBR3 cells were labeled with 10 μg/ml trastuzumab (Herceptin) (Roche) in 1% (w/v) BSA/PBS, after washing, as bridging antibody 4 μg/ml polyclonal rabbit anti-human IgG (code-Nr. A 0424, Dako) diluted in 1% (w/v) BSA/PBS was used. After the next washing protein-A gold 15 nm was used (PAG15, CMC, Utrecht, The Netherlands) 1:60 diluted in 1% (w/v) BSA/PBS to mark the location of the HER2 receptors. All washing steps were done with 0.1% (w/v) BSA in PBS.

2.9.3. Post-fixation

After labeling, the SKBR3 cells were fixed for 1 h at RT or overnight, or over-weekend at 4 °C in a modified Karnovsky fixative (Karnovsky, 1965), which was concocted as follows: 5 ml 4% (w/v) FA; 2 ml 0.4 M sodium cacodylate buffer pH 7.4, 1 ml 25% glutaraldehyde, 0.5 ml 5 mM $CaCl_2$, 0.5 ml 10 mM $MgCl_2$ and 1 ml distilled water for 10 ml fixative pH 7.4. After washing with 0.1 M sodium cacodylate buffer pH 7.4, SKBR3 cells were post-fixed for 1 h on ice in 1% (w/v) OsO_4 (w/v) + 1.5% (w/v) K_4FeCN_6 (w/v) in 0.065 M sodium cacodylate buffer pH 7.4.

2.9.4. Dehydration

After rinsing 8 times with distilled water the samples were dehydrated in an increasing series of Ethanol, i.e. 30%, 50%, 70%, 80%, 90%, 96% with each step two times 5 min, thereafter 100%, six times 5 min at RT. Anhydrous ethanol was made by 100 ml 96% Ethanol and 2 ml acidified 2,2-dimethoxypropane (DMP). Acidified DMP was made by adding 50 μl 37% HCl to 50 ml DMP (Muller and Jacks, 1975).

2.9.5. Embedment

Samples were infiltrated with a mixture of anhydrous ethanol-Epon 1:1 for 18 h; 1:2 for 8 h; 1:3 for 18 h, and subsequently infiltrated with pure Epon for 8, 18 and 6 h, each step with fresh pure Epon (Epoxy embedding medium, Araldite hardener 964, hardener MNA and accelerator DMP-30). The SKBR3 cells with supporting aclar were placed in the cavities of an embedding mould (Agar Scientific) with the cells positioned face up. The cavities were filled to the top with Epon, and polymerized for 72 h at 60 °C. Thereafter the specimen blocks were taken out of the mould, and the contour of the supporting aclar from each block was first scratched with the sharp corner of a single edged GEM razor blade, after which the aclar could be lifted up with the tip of a fine forceps and peeled off from the Epon block. Subsequently, all the blocks without the supporting aclar were put back into the mould with sample side up, and on each block a droplet of Epon was placed on the area, where the aclar was removed. Then the blocks were further polymerized for 24 h at 60 °C.

2.9.6. Ultra-microtomy

Epon sections of 50–55 nm were cut on a diamond knife (Diatome, Hatfield, PA, USA) with an Ultracut E ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were picked up with a loop and transferred to formvar (Fluka)-coated, carbon-stabilized copper grids (100 mesh hexagonal copper grids, Stork Veco B.V., The Netherlands) and post-stained with 7% (w/v) uranyl acetate (EMS) in 70% methanol (Merck) for 6 min, and with Reynolds' lead citrate (Lead nitrate, tri-Sodium citrate dehydrate, Merck (Reynolds, 1963) for 2 min. The thin sectioned and stained samples were viewed with a transmission electron microscope JEM 1010 (Jeol Electron Microscope 1010, JEOL (Europe) B.V., The Netherlands) at an acceleration voltage of 60 kV.

2.10. Scanning electron microscopy of immuno-gold labeled SKBR3 cells

Once the SKBR3 cells were immuno-gold labeled and post-fixated for 1 h on ice in 2% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer pH 7.4, the samples were transferred into specimen baskets after washing 8 times with Milli-Q water and subsequently dehydrated in an increasing series of ethanol as described in the section pre-embedment labeling above, and further processed for scanning electron microscopy. Samples were transported in the specimen baskets (Plastic capsules D 13 x H 18 mm fine mesh base, Leica) to a Leica CPD-300 critical point drying apparatus (Leica Microsystems, Vienna, Austria) with a CPD-chamber that was half filled with anhydrous ethanol and a 1/2-height-Teflon holder. The specimen baskets with samples were rapidly put into the CPD-chamber in a way that they were still submerged in anhydrous ethanol. Once this holder was filled with the samples, a 1/3-height-Teflon holder was put in the CPD-chamber and was filled with the remaining specimen baskets with samples. On top of this 1/3-height-Teflon holder, a 1/6-height-filler was placed and the chamber was further filled with anhydrous ethanol. After closing the CPD-chamber the samples were critical point dried according to the Leica CPD-300 manual with liquid CO_2 as the transitional fluid. We used the following program settings: Automatic On, Stirrer On and 100% CO_2 in, with speed slow and a delay of 120 s; exchange with speed 5 and cycles 12; CO_2 out, heat slow and gas out with speed 40%. The total CPD processing time was about 2 h and 10 min. The dried specimens were mounted on Aluminum stubs (agar scientific) containing a carbon adhesive (agar scientific), and were subsequently coated with 1 nm Pt by the use of a Leica ACE600 sputter coater according to the Leica ACE600 manual (Leica Microsystems, Vienna, Austria). Thereafter the samples were viewed in an XL30 scanning electron microscopy equipped with a field emission gun (FEI Europe, Eindhoven, The Netherlands) at an acceleration voltage of 5 kV and a WD of 2.8 mm.

3. Results

3.1. Effect of formaldehyde fixation on the binding of anti-HER2 nanobody

To evaluate the application of nanobodies in electron microscopy we used nanobody 11A4, which binds to cell surface-located HER2 with an affinity of 500 pM (Kijanka et al., 2013). In resting cells, HER2 is expressed predominantly at the plasma membrane as internalization of this receptor tyrosine kinase is blocked by Hsp90 (Bertelsen and Stang, 2014). During the course of these experiments we used trastuzumab, a humanized mAb binding to human HER2, as a positive control. Essential for the preservation of the cellular structure for EM is the fixation, which is usually done with aldehydes. As fixation with aldehydes might also affect the epitope, we first evaluated the effect of formaldehyde fixation on the binding of both the HER2-targeted nanobody and the mAb to HER2 expressed by SKBR3 cells. SKBR3 cells were incubated with different concentrations of nanobody or mAb either before or after fixation, and their binding was quantified by indirect fluorescence at 800 nm. Saturable binding to SKBR3 cells was observed for both nanobody and mAb with apparent affinities in the low nanomolar range. Surprisingly, the highest binding, both with respect to affinity (K_D) and B_{max}, was observed for labeling of samples fixed prior to nanobody or mAb incubation (Fig. 1A, B).

Both nanobody and antibody binding have off rates (k_{off}) between 10^{-3} and 10^{-5} s^{-1} resulting in a considerable dissociation within four hours, and as a result we expect to see a reduction in immuno-labeling efficiency seven days after the binding reaction. We quantified the effect of post-fixation on immuno-labeling of different concentrations of nano- and antibody seven days after labeling (Fig. 1C). Surprisingly, the reduction in immuno-labeling was not more than 30%. A significant improvement of immuno-labeling, the lowest decrease in signal, was observed in samples that were fixed prior to nanobody labeling and subsequently post-fixed using the same fixative (4% (w/v) formaldehyde) (Fig. 1C). Interestingly, such an effect was not observed when a bivalent antibody was used, which may be due to the reduced off rates as a result of bivalency (Fig. 1D). As expected, no effect was observed in samples that were first labeled, fixed and again post-fixed (Fig. 1C, D). In conclusion, optimal binding of both VHHs and mAbs is obtained when samples are first fixed, followed by immuno-labeling. A postfixation step is improving immuno-labeling performed with nanobodies.

3.2. Optimization for on-section immuno-gold labeling

The on-section labeling method of Tokuyasu cryosections is an ideal technology to label both intra- and extracellular proteins. However, this method is also challenging with respect to the occurrence of a-specific labeling. To obtain optimal immuno-gold labeling of cryosections, the background labeling should be minimized without hampering specific interactions at the same

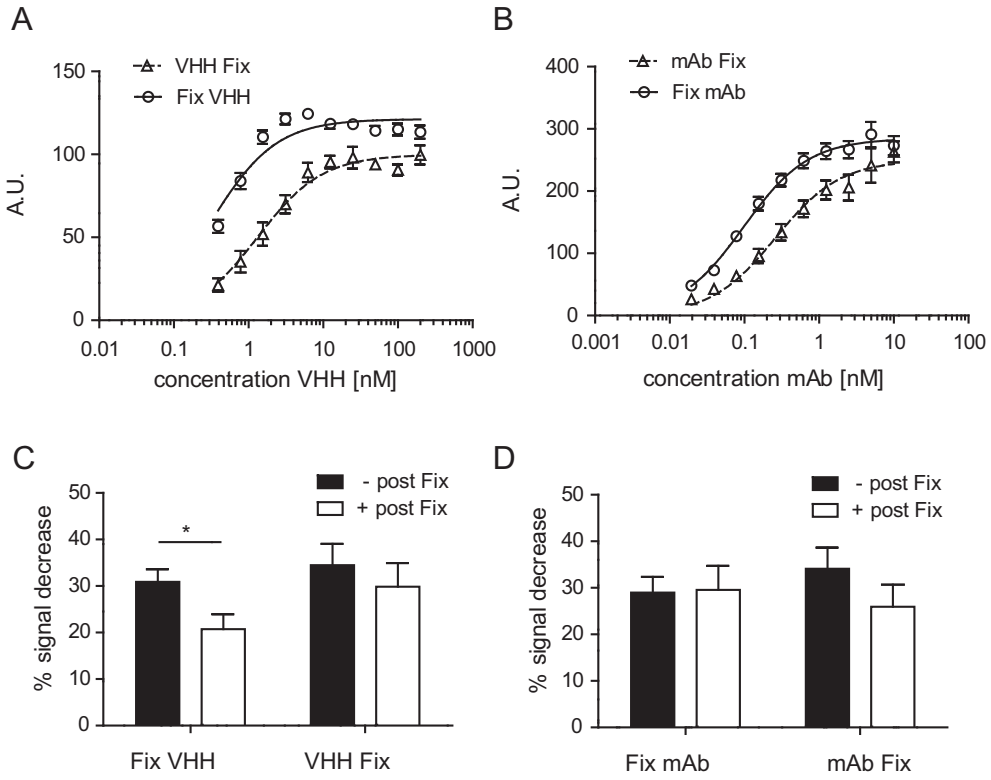

Fig. 1. Effect of formaldehyde fixation on the binding of 11A4 nanobody or trastuzumab A. SKBR3 cells were either fixed with 4% formaldehyde prior to or after immuno-incubations with increasing concentrations of VHH. Nanobody binding was detected by anti-VHH polyclonal serum and secondary antibody conjugated to fluorophore IRDye800CW. A.U, fluorescence intensity at 800 nm arbitrary units. (n = 3, each in triplicate, SEM: standard error of the mean). Curve fitting was performed with one-site specific binding, using Prism GraphPad. B. Effect of fixation with 4% formaldehyde prior or post mAb labeling analysed as described under A. C. SKBR3 cells fixed prior or post labeling with a saturating concentration of VHH 11A4 (12.5 nM) and subsequently post-fixed with 4% formaldehyde. Samples were stored for one week in the cold in PBS and binding was analysed using an Odyssey as described in M&M. (n = 3, each in triplicate, SEM). D. SKBR3 cells fixed prior or post labeling with 2.5 nM trastuzumab (mAb) and subsequently post-fixed and analysed as described under C.

time. We have evaluated the fixation and immuno-labeling conditions specifically for cryosections with immuno-fluorescence studies. Cells without HER2 expression, MDA-MD-231, and the HER2 expressing SKBR3 cells were fixed with formaldehyde and processed for cryosectioning according to the Tokuyasu method (Karnovsky, 1965; Muller and Jacks, 1975). Several blocking solutions commonly used for post-embedment labeling of antigens for TEM were analysed for specific immuno-labeling. Thick (500 nm) cryosections were incubated with different concentrations of HER2-targeted nanobody and the level of background staining was evaluated in wide field fluorescence microscopy. We present the results of two different blocking solutions: the standard bovine serum albumin (BSA) and a combination of cold water fish gelatin and acetylated bovine serum albumin (CFG/BSA-c) in PBS (Fig. 2A, B). No fluorescence was observed on sections that were blocked with either of the two solutions when the primary incubation was omitted (Fig. 2). This indicates that the fluorescent secondary antibodies do not cause a-specific binding under these conditions. In the presence of different concentrations of nanobody, BSA alone could not prevent a-specific binding sufficiently, which was found both on sections from HER2 positive (SKBR3) and negative (MDA-MB-231) cells. Even the lowest concentration of nanobody (1 μg/ml) resulted in considerable background staining of sections from MDA-MB-231 cells (Fig. 2A). In contrast, a blocking solution composed of a mixture of both CFG and BSA-c reduced the a-specific interactions on these two cell types considerably, even at high nanobody concentrations (10 μg/ml) (Fig. 2B). Much less background was present on SKBR3 cryosections, whereas the specific signal was retained, and hardly any background could be detected on the HER2 negative cells, especially when the lowest nanobody concentration was used.

In order to preserve the fluorescent signal for a longer time during storage at 4 °C we tested whether an additional post-fixation step could prevent dissociation of the immune complexes (Fig. 3). For this reason sections were or were not post-fixed upon incubation with secondary antibody (goat anti-rabbit-Alexa488) before DAPI staining and embedment in ProLong Gold. Especially, as can be seen at a concentration of 1 μg/ml 11A4, the fluorescence signal is better preserved in samples with the post-fixation step, which is in agreement with the data presented above (Fig. 1). Again, the post-fixation is not affecting the fluorescent signal obtained with the bivalent anti-HER2 monoclonal antibody trastuzumab (Fig. 3).

In the next step we evaluated the suitability of these fixation and blocking agents for nanobody-based immuno-gold labeling of cryosections (Fig. 4). To enable binding of gold particles to the tag-less anti-HER2 nanobody we used indirect labeling using rabbit anti-VHH serum followed by protein A-gold particles of 15 nm. Next to 1% BSA and 0.225% CFG / 0.1% BSA-c we also included 1% and 2% skimmed milk (FREMA) in PBS, 1% CFG, and 1% BSA-c. Employment of 1% BSA as blocking solution resulted in high levels of gold labeling in the cytoplasm, which is considered as a-specific, background labeling. This background staining was not diminished upon decrease of nanobody concentration used (from 10 μg/ml to 3 μg/ml) (Fig. 4). Application of a high concentration (1%) acetylated BSA resulted in a complete absence of

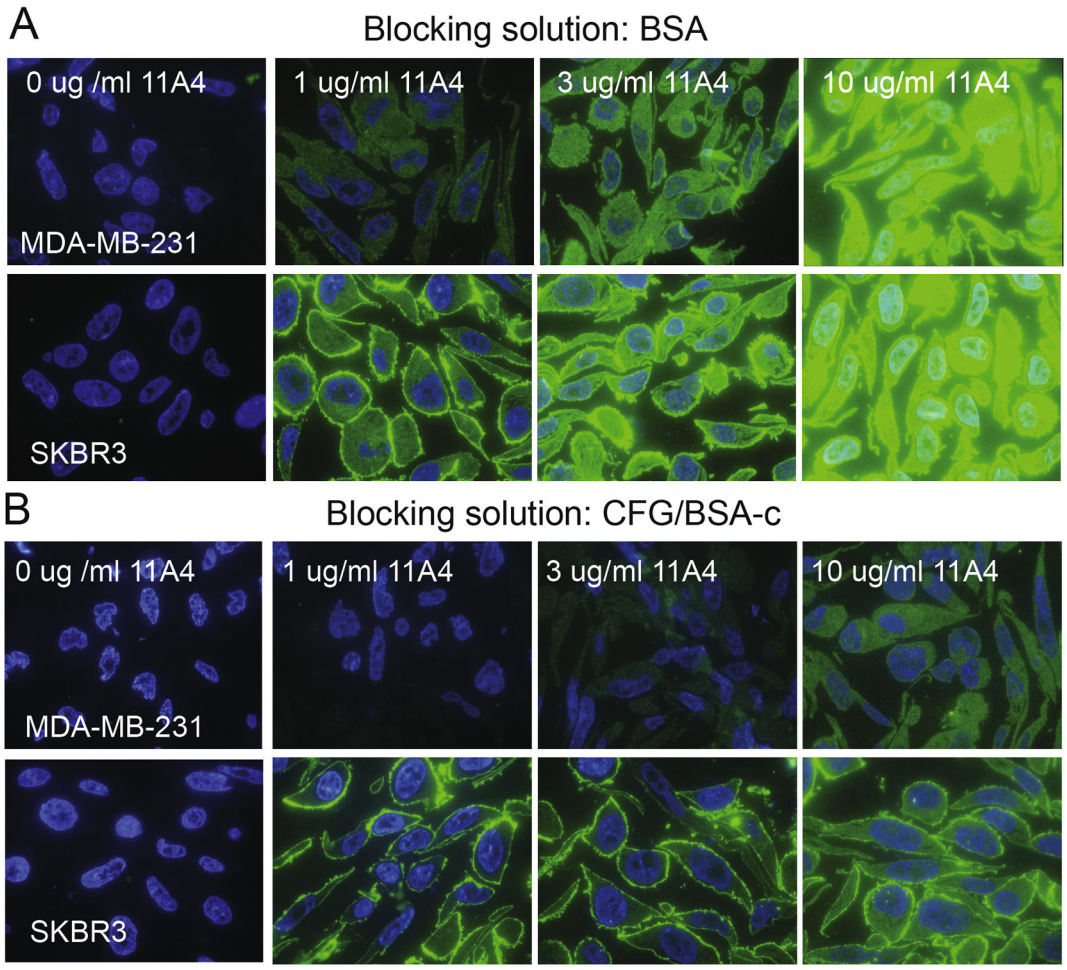

Fig. 2. Comparison of different blocking solutions by light microscopy. Thick cryosections of 500 nm of HER2-positive SKBR3 cells and HER2-negative MDA-MB-231 were incubated with different concentrations of anti-HER2 nanobody 11A4 (1, 3 and 10 μg/ml) in the presence of either (A) bovine serum albumin (BSA) or (B) a mixture of cold water fish gelatin and acetylated bovine serum albumin (CFG/BSA-c). Immune complexes were stained with secondary antibodies (Rabbit anti-VHH and Rabbit anti-Human IgG) and tertiary antibodies conjugated to Alexa 488 and nuclei were stained with DAPI. Sections were examined by wide field fluorescence microscopy.

background staining, but unfortunately also of specific HER2 labeling. Similar results were obtained with 1% and 2% FREMA (data not shown). Optimal results were obtained with the mixture of 0.225% CFG and 0.1% BSA-c. With 10 μg/ml of HER2-targeted nanobody still some background staining was present, however, after reduction of nanobody concentration to 3 μg/ml, this background staining was almost completely absent, while the specific label was preserved (Fig. 4). The specific HER2 gold labeling was predominantly present at the plasma membrane, particularly in membrane ruffles or filipodia. No gold particles were observed in the coated pits confirming previous data (Reynolds, 1963).

We next wanted to analyze the effect of the purification tag on immuno-gold labeling of cryosections. Purification tags such as the His-tag and the EPEA- or C-tag simplify the purification of nanobodies. Here we compared the binding of a tagless VHH, which was purified on basis of the protein A-binding property of this nanobody, and VHHs equipped with either the His- or EPEA-tag, which can be purified using Immobilized Metal Affinity Chromatography (IMAC) or the Capture Select C-tag affinity matrix respectively. A-specific gold labeling was again visible on sections labeled with the BSA blocking solution (Fig. 1S). When the CFG/BSA-c mixture was applied as blocking solution, no a-specific labeling was observed for the tagless, His- and EPEA-tagged nanobody (Fig. 5). However, His-tagged nanobodies show a less intense staining as less gold particles are visible at the membrane using similar nanobody concentrations. Finally, we tested the specificity of the 11A4-EPEA labeling by using a non-relevant nanobody that is binding to a organic dye (R2)(7). No labeling was observed on these sections by either of the two tested R2-EPEA concentrations (3 and 10 μg/ml) using the CFG/BSA-c mixture (Fig. 5). A modest backgroundlabeling for R2 was observed on sections using BSA (Fig. S1). These results further demonstrate the specificity of the 11A4-EPEA anti-HER2 nanobody and the effect of the CFG/BSA-c on the reduction of background labeling.

3.3. Nanobody-based pre-embedment immuno-gold labeling for TEM

The optimized labeling procedure was subsequently tested for pre-embedment labeling and examination using TEM. SKBR3 cells were incubated with either the tag-less anti-HER2 nanobody (11A4) or the monoclonal anti-HER2 antibody trastuzumab. Rabbit anti-VHH and protein A-gold was used to detect the nanobody while for trastuzumab detection we used rabbit anti-human IgG followed by protein A-gold. After the different incubations, cells were post-fixed and further processed for electron microscopy. Immuno-gold labeling was completely absent in control samples where VHH was omitted or cells were used lacking HER2 expression (MDA-MD-231) (Fig. 6A, B). Gold labeling of nanobody and mAb treated samples was observed for cells labeled before and after fixation (Fig. 6C–F). This result confirms that prior formalde-

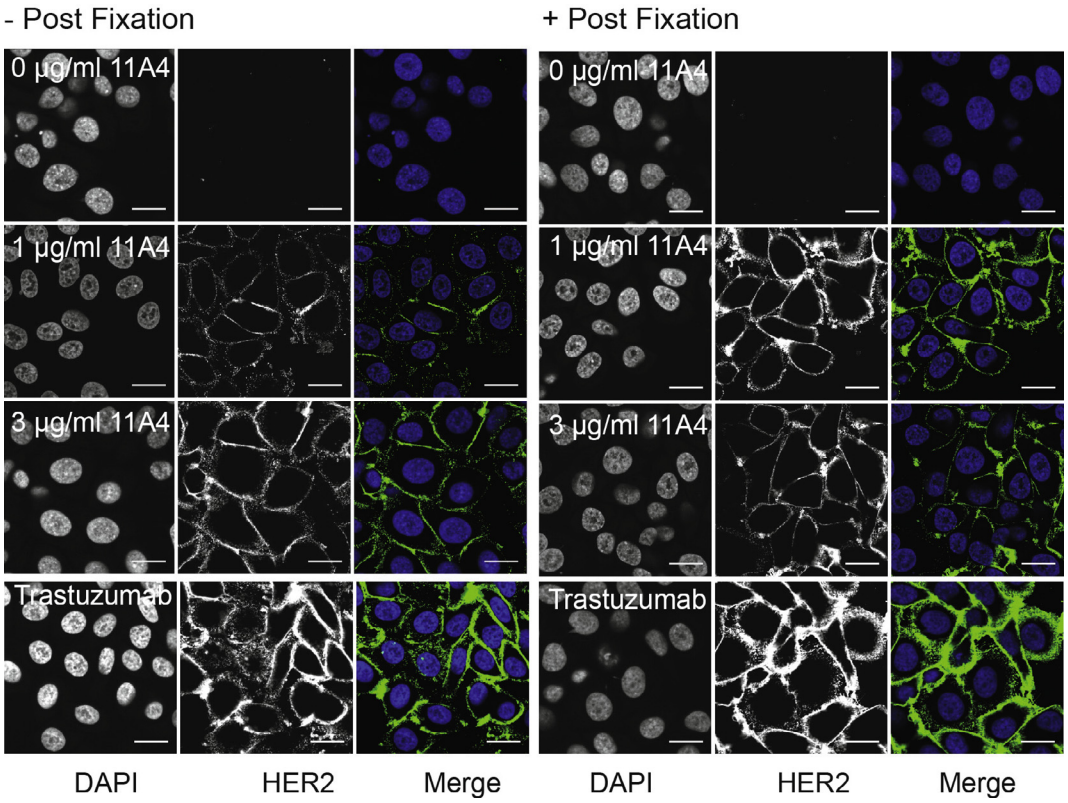

Fig. 3. Effect of post fixation on preservation of immuno-labeling of HER2 on thick Tokoyasu sections. Thick cryosections of 500 nm of HER2-positive SKBR3 cells were incubated with different concentrations of anti-HER2 nanobody 11A4 (1 or 3 µg/ml) in CFG- + BSA-c or 10 µg/ml trastuzumab in BSA. Immune complexes were stained with secondary and tertiary antibodies conjugated to Alexa 488 and nuclei were stained with DAPI. Cells were post-fixed in 4% (w/v) formaldehyde and imaged after 1 week storage at 4 °C.

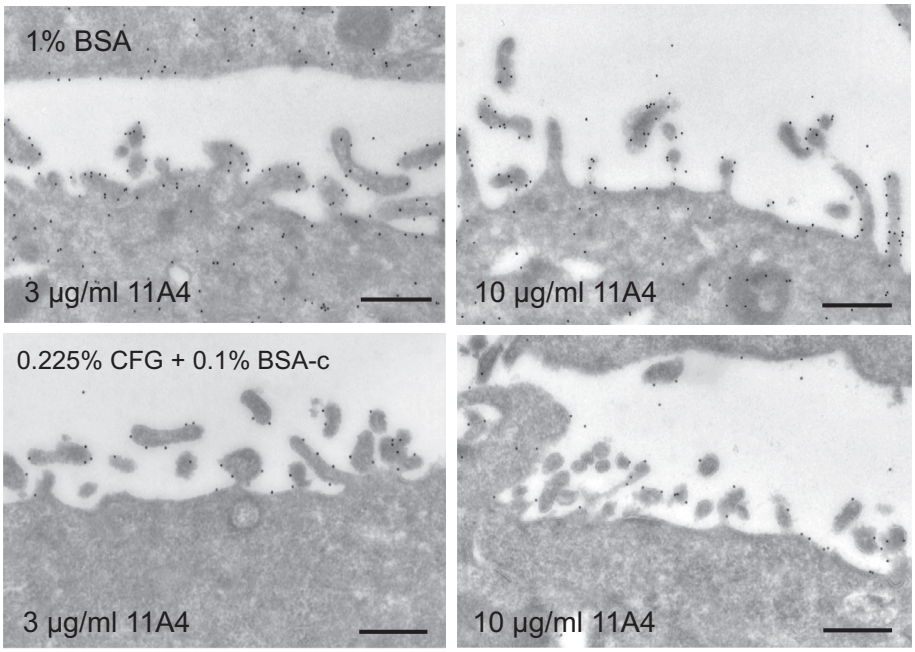

Fig. 4. Comparison of different blocking solutions used for Tokuyasu cryosections. SKBR3 cells were fixed with 4% (w/v) formaldehyde and processed for cryosectioning. Sections were blocked with either BSA or a mixture of cold fish gelatin and acetylated bovine serum albumin, followed by a labeling for HER2 with the anti-HER2 nanobody 11A4 at two different concentrations (3 and 10 µg/ml). Immune complexes were stained with rabbit anti-VHH/protein-A 15 nm gold particles. After post-fixation the samples were stained and examined with a transmission electron microscope as described in M&M. Scale = 500 nm.

hyde fixation does not affect the binding of either the HER2-targeted nanobody (11A4) or the mAb (trastuzumab). Gold labeling was particularly seen at the membrane ruffles or filopodia, which is in agreement with the cryosection labeling data.

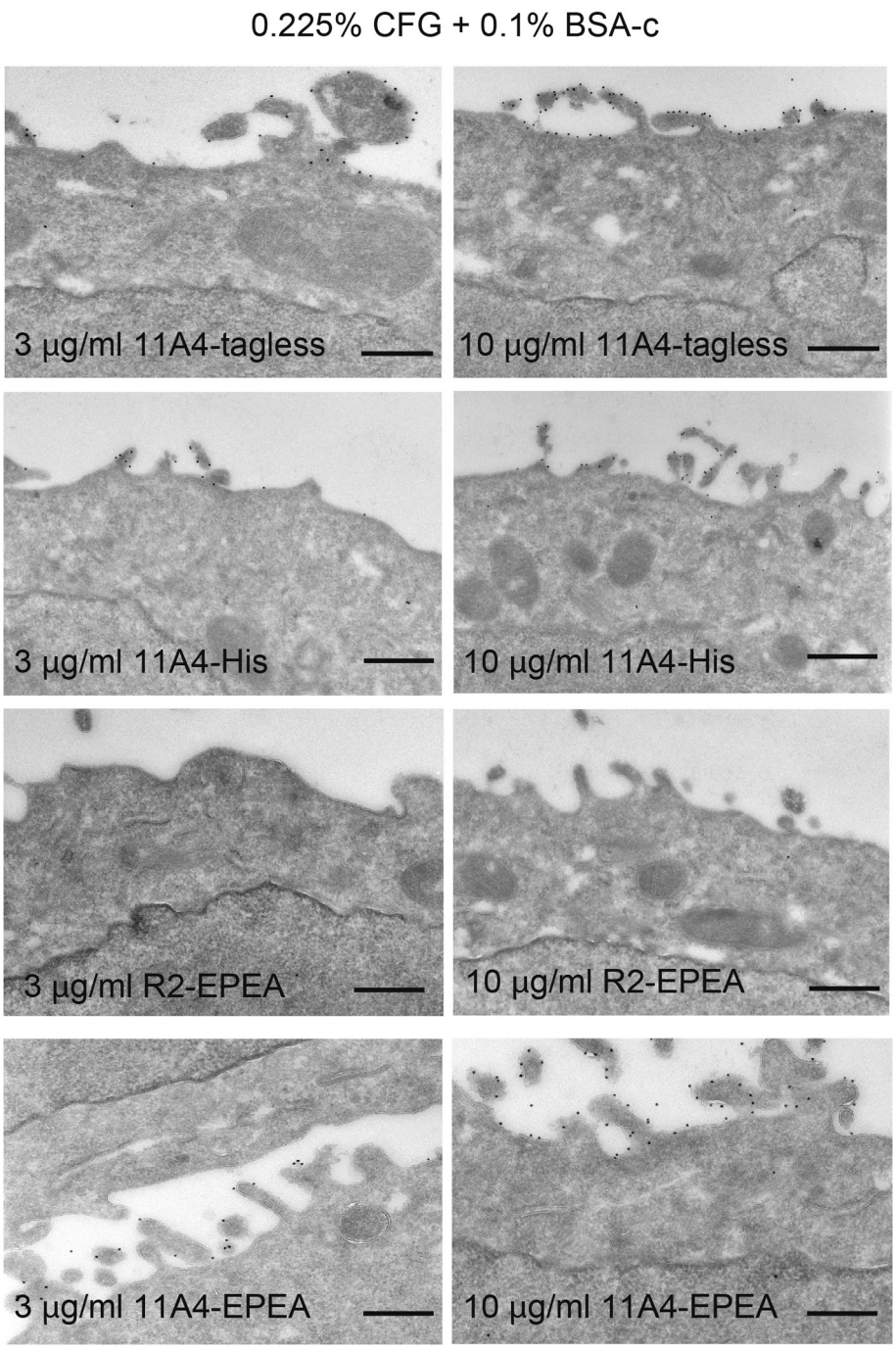

Fig. 5. Effect of purification tag on immunogold labeling of HER2. SKBR3 cells were fixed with 4% (w/v) formaldehyde and processed for cryosectioning. Sections were blocked with a mixture of cold fish gelatin and acetylated bovine serum albumin, followed by a labeling for HER2 with either the tagless anti-HER2 nanobody 11A4 or nanobody containing a C-terminal His- or EPEA-tag at two different concentrations (3 and 10 µg/ml). As control we used the non-relevant nanobody designated as R2 (Kijanka et al., 2013). Immune-complexes were stained with protein-A 15 nm gold particles. After post-fixation the samples were stained with uranyl acetate and examined with a transmission electron microscope as described in M&M. Scale = 500 nm.

3.4. Nanobody-based pre-embedment immuno-gold labeling for SEM

We finally tested the feasibility of HER2-targeted nanobodies for SEM applications using SKBR3 cells. In this experiment we again used the HER2-targeted mAb, trastuzumab, as a positive control. As a negative control the incubation with the nanobody was omitted. SKBR3 cells were fixed and immuno-gold labeling was performed using the optimal blocking mixture as described above (0.225% CFG/0.1% BSA-c). No gold-labeling was seen in the SEM pictures of cells that were not incubated with nanobody, but incubated with anti-VHH serum followed by protein A-gold (Fig. 7A). Immuno-gold labeling is clearly visible in case of cells labeled either with nanobody or trastuzumab (Fig. 7B and C). Interestingly, filopodia/membrane ruffles that are appearing at the side of the SKBR3 cells were heavily labeled with the nanobody-anti-VHH-protein A gold complexes when 15 nm gold particles were used (Fig. 7D).

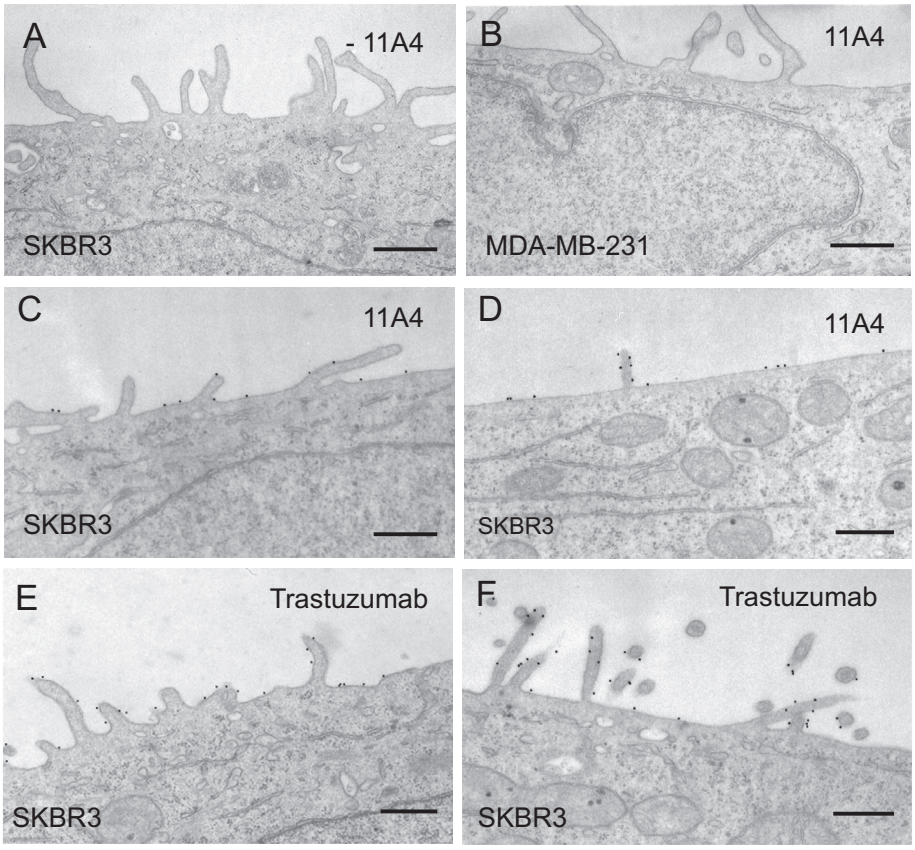

Fig. 6. Transmission EM of pre-embedment nanobody-based immuno-gold labeling of HER2 in SKBR3 cells. SKBR3 or MDA-MB-231 cells were fixed with 4% (w/v) formaldehyde and processed for immuno-labeling. As negative controls, primary antibody was omitted (A) or MDA-MB-231 cells lacking HER2 expression were used (B). SKBR3 cells were either incubated with anti-HER2 nanobody 11A4 (VHH) (C, D) or trastuzumab (mAb) (E, F) after (C, E) or before (D, F) formaldehyde fixation. Cells were post-fixed, dehydrated and embedded in Epon. Ultrathin sections were examined by transmission EM. Scale = 500 nm.

4. Discussion

In the present study we describe for the first time the successful employment of nanobodies for different immuno-EM applications. For these studies we used the previously developed HER2 targeted nanobody, 11A4, which binds specifically and with high affinity to the HER2 receptor present on the surface of breast cancer cells (Kijanka et al., 2013). Parameters that are affecting immuno-gold labeling were optimized and this resulted in an optimal procedure for the employment of the anti-HER2 nanobodies in immuno-gold labeling studies using both transmission and scanning EM.

Essential for the preservation of ultrastructure of cells and tissues is their fixation. The aldehydes that are used in most fixatives bind covalently to primary amines (in lysines) or thiols (in cysteins) within the HER2 molecule, which might render the antigen structure unrecognizable for mAb/nanobody. Formaldehyde is a commonly used fixative in EM applications that does not lead to much tissue shrinkage or distortion of cellular structure (Thavarajah et al., 2012). Fixation with formaldehyde performed before nanobody incubation did not affect the binding of either 11A4 or of trastuzumab to HER2, suggesting that both epitopes do not contain lysine or cystein residues. Also other nanobodies (e.g. anti-EGFR) were tested for their sensitivity for aldehyde fixation, revealing that binding of at least these nanobodies, were not affected by this type of fixation (data not shown). However, aldehyde sensitivity of nanobodies should be evaluated for each new nanobody, just as for antibodies.

To optimize the nanobody-based immuno-labeling, several blocking solutions were investigated among which were BSA, acetylated (BSA-c), cold water fish gelatin and skimmed milk. BSA is a globular protein that adsorbs a-specifically onto the specimen surface. During this process the BSA is suggested to unwind and more contact-spots with the specimen are made (Leunissen, 2006). However, some spots on the specimen surface are too small for globular BSA to bind and remain uncovered. This may explain the higher background staining obtained with BSA alone as a blocking agent. We therefore combined both CFG and BSA-c into the same blocking solution. The lysine and arginine residues of BSA-c have been acetylated to increase negative charge to facilitate molecule linearization and increase its hydroplethobic character. Such linearized BSA-c is able to cover the specimen surface more uniformly, decreasing the non-specific background (Leunissen, 2006). CFG has also been described to prevent a-specific binding. Best results, in terms of the lowest background labeling and highest specific staining, were obtained when the combination of CFG and BSA-c was employed.

A post-fixation step at the end of the labeling procedure was found to improve nanobody labeling intensity. No effect was seen with mAb labeling suggesting that the dissociation kinetics under the employed conditions of mAb labeling was less than the dissociation of nanobody. As this might be explained by the bivalency of the mAb, bivalent nanobodies might improve labeling density of nanobody-based immuno-gold labeling. On the other hand, the post fixation step could reduce signal loss sufficiently.

Purification of nanobody is usually done by affinity chromatography using Protein-A, positively charged metal ions as nickel (IMAC) or the Capture Select C-tag affinity matrix (when nanobody possesses EPEA tag). Under optimal blocking condition, specific

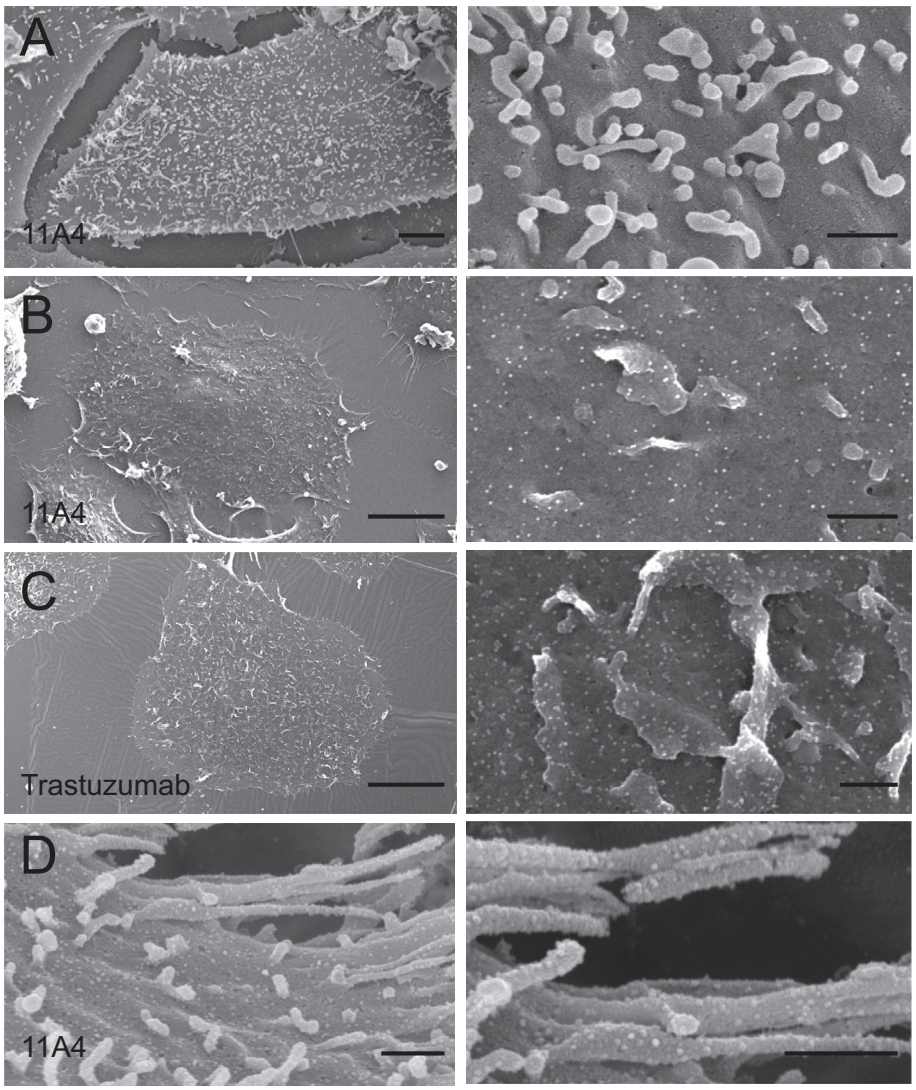

Fig. 7. Scanning EM of pre-embedment, nanobody-based immuno-gold labeling of HER2 in SKBR3 cells. SKBR3 cells were fixed, whether or not (A) incubated with anti-HER2 nanobody (B, D) or as positive control, with the anti-HER2 antibody trastuzumab (C). Immuno-labeling was performed in the presence of a mixture of cold fish gelatin and acetylated bovine serum albumin. Subsequently, cells were incubated with secondary antibodies and with protein A conjugated gold particles of 15 nm. Samples were critical point dried and examined by electron microscopy as described in Materials & methods. Scale: left panel: A: 1 μm, B, C: 0.5 μm, D: 0.2 μm; right panel: A: 250 nm, B-D: 100 nm.

labeling of all nanobodies was observed. However, for unknown reasons, less intense labeling, less number of gold particles was observed for the His-tagged nanobodies. Higher labeling can be expected from the use of a higher concentration of the anti-HER2 nanobody, but this will most probably coincide with higher background. Therefore, we prefer to avoid the use nanobodies that contain a His-tag.

Both immuno-fluorescence data as well as our TEM and SEM data show that HER2 is preferentially located at the membrane protrusions. The membrane protrusions on top of the cells, also indicated as top ruffles, did not reveal HER2 staining in all cases. However, the membrane ruffles at the side of the cells show intense staining of HER2. Similar data were previously reported by Hommelgaard et al. Hommelgaard et al. (2004) using conventional antibodies, who furthermore demonstrated the absence of HER2 in coated pits and coated vesicles.

In this paper we demonstrated the application of anti-HER2 nanobodies both in pre-embedment labeling (TEM and SEM) and with post-embedment labeling using Tokuyasu cryosections. The feasibility of this nanobody used for both pre- and post-embedment EM applications opens up a possibility to further improve the resolution of gold labeling in EM applications. So far the smallest targeting domain tested for EM applications has been the recombinant single chain variable fragment antibodies (scFv). Their small molecular mass (26 kDa) resulted in increased labeling efficiency in comparison to mAb (Malecki et al., 2002). Although, we have here presented indirect nanobody labeling, direct and site specific gold conjugation to the nanobody will be the next step in introducing nanobodies into the daily EM routine. We expect that direct labeling with nanobody-gold complexes will considerably improve the labeling resolution of EM as compared to conventional antibodies, because the size of the linker molecule will be reduced from 14.2 nm x 8.5 nm to the size of a nanobody ($3 \times 3 \times 4$ nm).

Acknowledgements

We would like to acknowledge Karin Vocking and Sylvia Denissen for their help with taking pictures of the fluorescent cryosections and Renée Scriwanek for the prints and scans of the TEM-negatives. This work was supported by the Center for Translational Molecular Medicine (CTMM) (grant 030-201) (M.K. and P. M.P.v.B.e.H.) and the KECK consortium (Prof. dr. Joe Gray, OHSU

Center for Spatial Systems Biomedicine, Oregon Health & Science University, Portland OR, USA) (E.G.v.D, and J.A.P.).

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.2017.05.008.

References

Faulk, W.P., Taylor, G.M., 1971. An immunocolloid method for the electron microscope. Immunochemistry 8 (11), 1081–1083.

Baschong, W., Stierhof, Y.D., 1998. Preparation, use, and enlargement of ultrasmall gold particles in immunoelectron microscopy. Microsc. Res. Tech. 42 (1), 66–79.

Xiaoying, Zhang., 2015. Gold nanoparticles: recent advances *in the* biomedical applications. Cell Biochem. Biophys. 72 (3), 771–775.

Hassanzadeh-Ghassabeh, G., Devoogdt, N., De Pauw, P., Vincke, C., Muyldermans, S., 2013. Nanobodies and their potential applications. Nanomedicine (Lond) 8 (6), 1013–1026.

Muyldermans, S., Baral, T.N., Retamozzo, V.C., et al., 2009. Camelid immunoglobulins and nanobody technology. Vet. Immunol. Immunopathol. 128 (1–3), 178–183.

Muyldermans, S., 2013. Nanobodies: natural single-domain antibodies. Annu. Rev. Biochem. 82, 775–797.

van Bergen en Henegouwen, P.M., Leunissen, J.L., 1986. Controlled growth of colloidal gold particles and implications for labelling efficiency. Histochemistry 85, 81–87.

Stierhof, Y.D., Schwarz, H., Frank, H., 1986. Transverse sectioning of plastic-embedded immunolabeled cryosections: morphology and permeability to protein A-colloidal gold complexes. J. Ultrastruct. Mol. Struct. Res. 97, 187–196.

Kijanka, M., Warnders, F.J., El Khattabi, M., et al., 2013. Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for image-guided surgery. Eur. J. Nucl. Med. Mol. Imaging 40 (11), 1718–1729.

Yarden, Y., 2001. Biology of HER2 and its importance in breast cancer. Oncology 61 (Suppl 2), 1–13.

Slot, J.W., Geuze, H.J., 2007. Cryosectioning and immunolabeling. Nat. Protoc. 2 (10), 2480–2491.

Tokuyasu, K.T., 1973. A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. 57 (2), 551–565.

Sobue, K., Fujio, Y., Kanda, K., 1988. Tumor promoter induces reorganization of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells. Proc. Natl. Acad. Sci. U. S. A. 85 (2), 482–486.

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27, 137A–138A.

Muller, L.L., Jacks, T.J., 1975. Rapid chemical dehydration of samples for electron microscopic examinations. J. Histochem. Cytochem. 23 (2), 107–110.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.

Bertelsen, V., Stang, E., 2014. The mysterious ways of ErbB2/HER2 trafficking. Membranes 4 (3), 424–446.

Thavarajah, R., Mudimbaimannar, V.K., Elizabeth, J., Rao, U.K., Ranganathan, K., 2012. Chemical and physical basics of routine formaldehyde fixation. J. Oral Maxillofac. Pathol. 16 (3), 400–405.

Leunissen JLM, de Plas PFEM: Optimised Immuno Labeling using AURION Blocking Solutions and AURION BSA-c™ Newsletter 1 (2006).

Hommelgaard, A.M., Lerdrup, M., van Deurs, B., 2004. Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. Mol. Biol. Cell 15 (4), 1557–1567.

Malecki, M., Hsu, A., Truong, L., Sanchez, S., 2002. Molecular immunolabeling with recombinant single-chain variable fragment (scFv) antibodies designed with metal-binding domains. Proc. Natl. Acad. Sci. U. S. A. 99 (1), 213–218.