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Development and application of protein-based probes for correlated microscopy

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DOI:
[10.33612/diss.147586577](https://doi.org/10.33612/diss.147586577)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
de Beer, M. (2020). *Development and application of protein-based probes for correlated microscopy*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.
<https://doi.org/10.33612/diss.147586577>

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Development and application of protein-based probes for correlated microscopy

Marit de Beer
2021

The research presented in this thesis was performed at the Department of Biomedical Sciences of Cells and Systems, University Medical Center Groningen (UMCG), University of Groningen (RUG).

This PhD project was financially supported by *Stichting Techniek en Wetenschap* (STW) as part of microscopy Vallley project (12718)

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Marit de Beer

ISBN: 978-94-641-9074-8

Printing of this thesis was supported by:

Graduate School of Medical Sciences (GSMS) at the University of Groningen

University Medical Center Groningen (UMCG)

University of Groningen (RUG)

Nederlandse vereniging voor microscopie (NVvM)

umcg

**university of
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Netherlands Society
for Microscopy

Printed and published by: Gildeprint, www.gildeprint.nl

Cover design: Roos Adolfs

Lay-out: Marit de Beer

**rijksuniversiteit
 groningen**

Development and application of protein-based probes for correlative microscopy

Proefschrift

ter verkrijging van de graad van doctor aan de
 Rijksuniversiteit Groningen
 op gezag van de
 rector magnificus prof. dr. C. Wijmenga
 en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

Woensdag 28 april 2021 om 16.15 uur

door

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geboren op 4 januari 1991
 te Tilburg

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Chapter 1

General introduction and thesis outline

General introduction

The building blocks of life, including proteins, are organized at specific localizations within the cells, tissues and the human body. Visualization of these proteins with microscopy is essential to gain insight in their function both in health and disease. The imaging of proteins depends on probes, that can be either affinity-based or genetically encoded. Affinity-based probes use antigen-binding proteins to detect the protein of interest. The most commonly used antigen-binding proteins are immunoglobulin G (IgG) antibodies, and are often used to label endogenous proteins. For visualization in light microscopy (LM), IgGs are conjugated to tags like enzymes or fluorophores^{1,2}. In genetically encoded probes, a direct genetic fusion is made between protein and tag, limiting the visualization to exogenous proteins. Genetically encoded probes got a boost after the green fluorescent protein (GFP) was engineered for cell biology³⁻⁵. The direct binding between protein of interest and GFP made live-cell fluorescence LM imaging possible, which was further developed by using multi-colors for multiple target imaging⁶⁻⁸. For both genetically encoded and affinity-based probes in LM, the disadvantage is the resolution gap between the protein size ($\sim 0.1\text{-}10\text{ nm}$) and the diffraction limit of light ($\sim 250\text{ nm}$). The resolution of LM can be improved by using super-resolution microscopy (SRM), to achieve resolutions up to $\sim 20\text{ nm}$ ^{9,10}. The disadvantage however for both SRM as well as LM, is the lack of structural context of the unstained proteins.

The ultrastructural context of cells can be visualized with electron microscopy (EM) along with high resolution ($\sim 0.5\text{-}5\text{ nm}$)¹¹. In EM protein identification is, like in LM, probe dependent. Here, affinity-based probes often are conjugated to gold nanoparticles and genetically encoded EM probes are based on peroxidases. Yet, EM is limited in multiple target imaging and is restricted to fixed samples. Therefore, correlative LM and EM (CLEM) is developed to combine LM or SRM with EM to match the resolution of probe and protein^{12,13}. Probes visible in both modalities enables live-cell and/or multi-color fluorescence imaging followed by ultrastructural context examination based on the electron density of the probe.

Several of the above mentioned techniques have recently been rewarded with a Nobel prize^{5,9,11} and are implemented by many researchers over the world, indicating their high applicability and reproducibility. Reproducibility is key for both technique development and fundamental research. Nowadays several studies showed large numbers of irreproducible studies¹⁴⁻¹⁷. Multiple causes have been implied, like insufficient details in method section¹⁸ and misidentification of cell lines¹⁹⁻²¹. Visualization of proteins using IgGs also can lead to alternating data, because IgGs can have a batch-to-batch variation. IgGs are multi-domain proteins and depend on recombination²²⁻²⁴. In research, IgGs are the most used antigen binding protein and can be produced as mono- or polyclonal IgG^{25,26}. Monoclonal IgGs are generated by a single, immortalized cell clone, while polyclonal IgGs are generated by a pool of cells. Multiple studies showed alternating results using commercially available IgGs caused by (i) binding to only the wrong target, (ii) cross-reactivity with other proteins, or (iii) loss of binding ability to the target²⁷⁻²⁹.

Single-domain antigen-binding proteins like nanobodies can improve the reproducibility of affinity-based probes. Nanobodies are $\sim 15\text{ kDa}$ proteins derived from heavy-chain only Camelidae antibodies³⁰⁻³². This single-domain protein is not dependent on recombination, and therefore once the cDNA is known, a homogeneous protein population can be produced.

Genetic modifications can easily be added to a single-domain protein to add the cDNA of fluorescent and/or EM suitable probes for microscopic visualization.

The aim of this thesis is to improve protein detection in CLEM by novel multi-protein domain probe development. The new probe, based on a nanobody domain for targeting, allows for the combination of (live-cell) light microscopy, using a fluorescent module, imaging in large fields of view and subsequently have detailed ultrastructural context with high resolution in EM by enzymatic generation of an electron-dense precipitate. These probes have been implemented to define how extracellular vesicles deliver their cargo in target cells.

Thesis outline

Chapter 2 provides an overview of nanobody technology implemented in microscopy. Nanobodies are ten times smaller than antibodies, which improves label efficiency, penetration depth, live-cell imaging and resolution. They can be visualized in LM via chemical tagging with fluorescent dyes or genetic fusion with fluorescent proteins and in EM using gold or peroxidases. Different tools are described as guidelines for implementing nanobody technology for own research topics.

A novel nanobody-based probe called FLIPPER-body, was generated and described in **Chapter 3a**. The FLIPPER-body consists of a nanobody, a fluorescent protein and a peroxidase providing the probe with antigenicity, fluorescence and electron density, respectively. Here, we have shown a proof-of-concept targeting intracellular GFP or extracellular growth factor receptors. The labeling showed in both modalities, LM and EM, was highly efficient. Furthermore, the probe is designed such that the individual modules are easily switched to the researchers own needs. A disadvantage of the probe is the compromised ultrastructure, when targeting intracellular proteins, as a result of permeabilization. Permeabilization agents dissolve the lipids of in the cellular membranes, but this facilitates intracellular access of the probes. Therefore, a method of labeling intracellular targets, free of permeabilization was pioneered using FLIPPER-body and is described in **Chapter 3b**. This method showed that cells are permeable, after mild fixation, for small protein probes like FLIPPER-bodies, resulting in an efficient labeling but further optimization is needed to get the best preserved ultrastructure.

In **Chapter 4**, cytosolic expressed fluorescent nanobodies (fluobodies) were used to visualize cargo release in extracellular vesicle (EV) biology using CLEM. Membrane-bound GFP, inside EVs, is recognized by the cytoplasmic anti-GFP fluobody once the cargo is exposed to the cytoplasm, suggesting that membrane fusion occurs. These membrane fusion events occurred in maturing, LAMP1-positive, endosomes, that resulted in cytoplasmic cargo exposure. Additionally, this chapter shows that combining cytosolic probes with multiple microscopic techniques can lead to novel insights in cellular mechanisms.

In **Chapter 5**, we attempt to develop probes for colorEM, to increase the label palette in EM and thereby aid unbiased examination of EM data. ColorEM adds the elemental composition of the reconstructed EM image per pixel, by the collection of X-rays in a Scanning EM equipped with an energy dispersive X-ray spectroscopy (EDX) detector. Here, the use of antibodies conjugated to rare-earth heavy-metal polymers were investigated. Although the elements were detected on pure spotted antibodies, there were not enough

X-rays collected in labeled samples to visualize the localization of the antibodies in a cellular environment. Overall, we conclude that the technique needs further development, in both detection efficiency as well as in probe development.

Chapter 6 provides a summary, general discussion and future perspectives of the thesis.

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Chapter 2

Nanobody-based probes for subcellular protein identification and visualization

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Frontiers in Cellular Neuroscience (2020) 14:573278
doi: 10.3389/fncel.2020.573278

Abstract

Understanding how building blocks of life contribute to physiology is greatly aided by protein identification and cellular localization. The two main labeling approaches developed over the past decades are labeling with antibodies such as immunoglobulin G (IgGs) or use of genetically-encoded tags such as fluorescent proteins. However, IgGs are large proteins (150 kDa), which limits penetration depth and uncertainty of target position caused by up to ~25 nm distance of the label created by the chosen targeting approach. Additionally, IgGs cannot be easily recombinantly modulated and engineered as part of fusion proteins because they consist of multiple independent translated chains. In the last decade single domain antigen binding proteins (e.g. nanobodies) are being explored in bioscience as a tool in revealing molecular identity and localization to overcome limitations by IgGs. These nanobodies have several potential benefits over routine applications. Because of their small size (15 kDa), nanobodies better penetrate during labeling procedures and improve resolution. Moreover, nanobodies cDNA can easily be fused with other cDNA. Multidomain proteins can thus be easily engineered consisting of domains for targeting (nanobodies) and visualization by fluorescence microscopy (fluorescent proteins) or electron microscopy (based on certain enzymes). Additional modules for e.g., purification are also easily added. These nanobody-based probes can be applied in cells for live-cell endogenous protein detection or may be purified prior to use on molecules, cells or tissues. Here, we present the current state of nanobody-based probes and their implementation in microscopy, including pitfalls and potential future opportunities.

Keywords: Nanobody, Chromobody, Fluobody, Probes, Light microscopy, Super-resolution microscopy, Electron microscopy, Tagging

Introduction

Defining protein identity and visualizing protein localization is fundamental in biology. Uncovering dynamics of protein localization and function were boosted when green fluorescent protein (GFP) and other fluorescent proteins (FPs) were developed and used to tag proteins of interest¹⁻³. Advantages of these chimeric fusion proteins include the lack of distance between protein of interest and label, thereby improving the resolution, as well as the specificity of labeling derived from the genetic fusion. Disadvantages include modification of the target protein, with the consequence that unmodified endogenous proteins cannot be studied². To detect endogenous proteins, immunolabeling using antibodies (immunoglobulins, mostly of the IgG isotype; IgGs) conjugated with small fluorophores are typically applied. However, for intracellular targeting IgGs require plasma membrane permeabilization leading to a damaged ultrastructure⁴. Furthermore, IgGs are large (~150 kDa; ~14 nm long; **Table 1**). This may result in a distance greater than 25 nm between target and label in indirect conventional immunolabeling, the so-called linkage error^{5, 6}. In addition, IgGs are multidomain proteins which require post-translational modifications⁵ and therefore preclude routine controlled genetic modification and modular expression in conjunction with e.g. GFP.

Nanobodies (see **Box 1** for terminology) are single variable domains of heavy-chain only antibodies (hcAB) derived from Camelidae species^{5, 7-9}, but do not compromise in the binding-affinity compared to IgGs, due to its complementarity-determining region (CDR) organization^{5, 10, 11}. Nanobodies have been explored since 2006 as labeling tools in light microscopy (LM)¹², because of the several potential advantages of nanobodies over other labeling techniques. Nanobody-mediated targeting for protein identification is more precise than IgG targeting, as nanobodies are only ~15 kDa with a diameter of 2-3 nm (**Table 1**) and can be encoded by a relative short stretch single cDNA of 360 base pairs^{9, 13, 14}. This cDNA can genetically be fused to FPs cDNAs for intracellular (live-cell) imaging or tags can be added for

Box 1 | Nanobody terminology

Nanobody

Synonyms: single domain antibody (sdAB), variable domain of heavy-chain only antibody (VhH), nAbs.

Small antigen binding protein, derived from heavy-chain only antibodies (hcAB). These are produced in cell culture or in bacteria.

Intrabody

The cDNA of this probe is expressed in the cells for intracellular antigen targeting.

Chromobody

Synonyms: ffluobody, fluorescent nanobody.

Genetic fusion of intrabody and fluorescent protein. Direct visualization with microscopy is possible.

Labeled nanobody

Synonym: ffluobody, fluorescent nanobody.

Nanobodies protein purified and tagged in vitro with e.g. a chemical dye.

purification and chemical modifications. Like IgGs, customized nanobodies can be created against a protein of interest and the cDNA can be shared free of charge, as opposed to IgGs^{15, 16}. Here, an overview is given about the past and potential future of nanobody application in microscopy.

1. Nanobodies in light microscopy

Nanobodies can be expressed in cells conjugated to a detection module (like GFP) to target endogenous intracellular proteins, or they can be expressed, purified and then applied in immunolabeling resembling traditional immunofluorescence approach. Conventional immunolabeling is performed using IgGs, but for an improved penetration nanobodies can be used as an alternative¹⁷. The improved penetration of nanobodies is illustrated in nuclear labeling of anti-GFP labeling targeting Histone2B (H2B)-GFP. Note that, in an equal labeling time, the nanobodies are colocalizing in the nucleus with the GFP, whereas the IgGs are mainly localized in the cytoplasm (**Figure 1A**). Though the anti-GFP is the best characterized and most used nanobody to date (**Table 2**), nanobodies are also used for visualizing endogenous proteins (**Figure 1B, Table 2**). Finally, nanobodies can be applied for live-cell imaging, both as purified proteins typically targeting extracellular antigens or being expressed from its introduced cDNA and targeted to intracellular antigens (**Figure 1C**)^{12, 18}.

Table 1 | Overview of different probes used in microscopy.

	IgG	FP	Nanobody	Chromobody	Fluorescent nanobody	Apex2-nanobody	FLIPPER-body
Reagent
Synonyms	-	-	VhH, sdAB	Intrabody	Fluobody	-	
Size (kDa)	150	27	15	42	15	43	86
Cellular expression							
Endogenous detection							
Live-cell LM	*		*				
EM	*		*				
Color code	Positive		Moderate			Negative	
* requires secondary labelling step
IgG nanobody FP Chemical dye Peroxidase

Figure 1 | Nanobodies improve penetration, detect endogenous proteins and are applicable in live-cell imaging. (A) Anti-GFP nanobody labeling (mCherry and peroxidase fused) and IgG labeling in H2B-GFP expressing cells. Cells permeabilized for 5 minutes with 0.1% Triton before labeling. Nanobodies and primary and secondary antibodies incubated for 1hr each. Note the colocalization between GFP and mCherry (nanobody), most prominently in the low-expressing cells, while Alexa Fluor 594 (IgG) mainly localizes in the cytoplasm. (B) High HER2 expressing cells, SkBr3, labeled with nanobodies targeting HER2. Overlay of nanobody fluorescence and EM image. Note the positive labeling at cell-cell contact sites. (C) SkBr3 cells with GFP at the extracellular site of the plasma membrane. Anti-GFP nanobody (mCherry and peroxidase fused) were added to live-cells. After 30 min staining, the signal was saturated. Bars: 10 μ m. A and B are reproduced from de Beer et al., 2018¹⁹, C is own data.

1.1 cDNA delivery of chromobodies for intracellular targeting

The first nanobody-based visualization of intracellular targets was achieved by the fusion to FPs ('chromobodies'¹²; **Table 1**), being expressed in the target cells. Since then more nanobodies were developed targeting proteins inside cells (**Table 2**, delivered as cDNA). These intracellular chromobodies have been applied in small organisms like *Danio rerio*²⁰, *Drosophila melanogaster*²¹, *Caenorhabditis elegans*²² and *Toxoplasma gondii*²³ allowing live-cell and intravital microscopy. Intracellular expression of chromobodies in mice was successfully achieved by infecting the mice with adeno-associated viral particles containing the coding sequence of the chromobody (**Figure 2A**)²⁴. Importantly, these chromobody approaches opens the opportunity for intravital imaging of endogenous proteins.

Next to defining protein localization, chromobodies can also relay functional changes in cells by fusing the nanobody to a fluorescent sensor for e.g. Ca^{2+} or pH²⁵. For instance, anti-GFP nanobodies are fused to a Ca^{2+} sensor targeting GFP labeled mitochondria²⁶. The nanobody facilitates the Ca^{2+} sensor to be in close proximity of the mitochondria to allow for Ca^{2+} dependent fluorescence readout. This results in the imaging of the local Ca^{2+} concentrations upon different stimuli. In the same study, anti-GFP nanobodies were conjugated to a SNAP-tag, a 20 kDa protein, modified from the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase²⁷. The SNAP-tag is used to recruit a chemical dye, which facilitates live-cell imaging for Chromophore-Assisted Light Inactivation (CALI)²⁸: laser-induced subcellular destruction of a protein of interest. Thus nanobodies allow precise molecular targeting and enable analysis of the function of biomolecules, as well as precise protein manipulation with CALI allowing a direct cause/consequence study in living cells.

Fluorescence signal from unbound chromobodies

Despite the success of chromobodies as intracellular probes, a disadvantage is their continued

Table 2 | Nanobody implemented in microscopy – An overview of targets that have been visualized using nanobodies and microscopy. Nanobodies are delivered to the cell as purified proteins or via cDNA. LM: Fluorescence light microscopy; EM: Electron microscopy; SR: Super resolution fluorescence microscopy; CLEM: Correlated LM/EM.

<i>Target</i>	<i>Delivered as</i>	<i>Technique</i>	<i>Reference</i>
Actin	cDNA or Protein	LM / SR	20, 23, 24, 84-88
Active β 2-Ars	cDNA	LM	89
Alexandrium Minutum	cDNA	LM	90
ALFA-tag	cDNA or Protein	LM / SR	59
AMIGO-1	cDNA	LM	91
Amyloid β	Protein	LM	92
Arabidopsis Thaliana	Protein	EM	93
ARTC2	Protein	LM	94, 95
ATP7B	cDNA	LM	96
bacteriophage p2	Protein	EM	97
BC2-tag	Protein	LM / SR	45, 56
β -catenin	cDNA or Protein	LM	54, 98
BFP	Protein	SR	99
CapG	cDNA	LM	32, 100, 101
CD11b	Protein	LM / CLEM	17, 102-105
CEA	Protein	LM	106, 107
C.Jejuni	Protein	LM	108
Clostridium Difficile toxin	Protein	LM	109
Cortactin	cDNA	LM	34, 54, 110, 111
Ebolavirus	Protein	LM	112, 113
EGFR	Protein	LM / CLEM	19, 114-122
Eps15	cDNA	LM	123
Extracellular vesicles	Protein	EM	124
Fascin	cDNA	LM	111, 125
FGFR1	cDNA	LM	126
γ -H2Ax	cDNA	LM	127
GPCR	cDNA	SR	128
Gelsolin	cDNA	LM	32, 100, 101, 129
Gephyrin	cDNA	LM	91
GFAP	Protein	LM / CLEM	17, 130
GFP / YFP	cDNA or Protein	LM / SR/ CLEM	12, 18, 19, 21, 22, 25, 29-31, 36, 46, 47, 50, 52, 53, 55, 65-67, 75-77, 86, 99, 125, 130-168, 200

(Continued)

Table 2 | Continued

<i>Target</i>	<i>Delivered as</i>	<i>Technique</i>	<i>Reference</i>
Gp41 (HIV)	cDNA	LM	61, 169
H2A-H2B	cDNA	LM	170
HER2	Protein	LM / CLEM	19, 43, 44, 72, 106, 171-174
HIF-1 α	Protein	LM	175
HIV-1	cDNA	LM / SR	176
Heterochromatin Protein 1 α	cDNA or Protein	LM	86
Homer1	cDNA or Protein	LM / SR	91
Human Neonatal Fc Receptor	Protein	LM	177
Huntingtin	cDNA	LM	178, 179
IGFBP7	Protein	LM	180, 181
IRSp53	cDNA	LM	91
Lamin	cDNA or Protein	LM / SR	12, 47, 55, 182
L-plastin	cDNA	LM	101, 183
LY-6C/6G	Protein	CLEM	17
Marburgvirus	Protein	LM	112, 113
MHC II	Protein	LM	102-104
Mouse IgG	Protein	LM / SR	184
NFT2	cDNA	LM	32
NTA domain cortactin	cDNA	LM	34
Nup35	Protein	SR	185
Nup37	Protein	SR	185
Nup85	Protein	LM / SR	186
Nup93	Protein	LM / SR	186, 187
Nup98	Protein	LM / SR	186, 187
Nup155	Protein	LM / SR	186
n-WASP	cDNA	LM	33
p53	cDNA or Protein	LM	86
PARP1	cDNA	LM	188
PCNA	cDNA	LM	20, 189, 190
PepTag	cDNA	LM	60
PFR1	Protein	LM	191
POM121	Protein	SR	185
PSMA	Protein	LM	192
Rabbit IgG	Protein	LM / SR	184

(Continued)

Table 2 | Continued

Target	Delivered as	Technique	Reference
RFP / mCherry	cDNA or Protein	LM / SR / CLEM	18, 25, 53, 67, 76, 77, 86, 99, 142, 148, 149, 152, 166
SAPAP2	cDNA	LM	91
SNAP-25	Protein	LM / SR	193
Survivin	cDNA	LM	11
Syntaxin 1A	Protein	LM / SR	193
Tau(phosphorylated)	Protein	LM	92
Tubulin	cDNA or Protein	LM / SR	6, 86, 194
VEGFR	cDNA	LM	195
Vimentin	cDNA or Protein	LM / SR	55, 196, 197
VSG	Protein	LM	58
Vsig4	Protein	LM	198
Vy9Vδ2-T cell	Protein	LM	199

presence. Chromobodies fluoresce whether or not they bind to their target, as opposed to immunolabeling techniques that include multiple wash-out steps for unbound reagents or genetic fusions between target and fluorescent proteins. To reduce the signal from non-bound chromobodies, conditionally stable chromobodies have been developed²⁹. These modified anti-GFP chromobodies are instable and rapidly degraded by the proteasome. When stabilized, however, these chromobodies will bind their target and consequently will no longer be degraded. Indeed, engineering and application of conditionally stable anti-GFP chromobodies resulted in a reduced background fluorescence²⁹. The mutations in the genetically modified nanobodies are highly conserved within nanobodies and therefore the switching from stable to instable nanobodies is generically applicable.

Non-targeted fluorescence can also be reduced by enhancer nanobodies³⁰. When the enhancer nanobodies bind with GFP, it increases the fluorescence and stability of the GFP. Enhancer nanobodies were also applied in a method to track single molecules in live-cell imaging³¹. Here, an array of nanobodies is fused to the protein of interest, and expressed in cells with cytosolic monomeric GFP. Upon GFP binding to the array of nanobodies, the GFP molecules increase in fluorescent intensity, resulting in an increased signal-to-noise ratio. This binding can in the microscope be seen as a single dot, that represents a single protein of interest. In conclusion, the signal-to-noise ratio of chromobodies can be improved by degrading unbound chromobodies or by enhancing the fluorescence of bound FPs, which both will be beneficial in the detection of proteins.

Delocalization of targeted proteins

Modifying cellular systems, in any way, may of course result in altered biology⁴. Interactions between endogenous proteins and ectopically expressed chromobodies can potentially influence the localization and function of the protein of interest. Altered localization for

Figure 2 | Nanobodies delivered for intracellular live-cell imaging. (A) Chromobodies cDNA loaded in e.g. adeno-associated viral particles (AAV). These viruses are used to infect cells in culture or in animals e.g. mouse. (B) Magnetic optical dimerization tool exists of two nMagHigh1 and pMagHigh1. Here, the nMagHigh1 is fused to nanobody fragment containing CDR1/2 and pMagHigh1 is fused to nanobody fragment containing CDR3. Upon stimulation of light, the magnetic tool paired together, resulting in restoring the nanobody. (C) Fluorescent nanobodies in e.g. oligomers can be taken up via endocytosis. Here, when endosomal rupture is induced, the free fluorescent nanobodies can bind to their target. (D) Microfluidic cell squeezing for temporary cell permeabilization. Purified fluorescent nanobodies are present in the medium, and diffuse into the accessible cytoplasm. (E) MoonTag: array of 24 peptide sequence repeat allows visualization of single molecules directly following translation by signal amplification. Chromobodies accumulate at the peptide chain. Major inspiration for this cartoon is from ^{39, 47, 55, 61}.

some proteins by introducing nanobodies is shown in a co-expression experiment (**Figure 3**). GFP-chimeras targeted to the nucleus or endoplasmic reticulum (ER) did not show altered localization when GFP-nanobodies were co-expressed (**Figure 3A, B**). However, chromobodies did alter the localization of GFP-fused lysosomal LAMP1 and GFP-fused connexins (Cx43 and Cx36). Normally, Cx43 and Cx36 localize at cell-cell contact sites, but chromobodies lead to their ER accumulation. Similarly, LAMP1-GFP together with chromobodies lead to an increased ER localization. Binding of nanobodies to their target during or post-translationally may disrupt proper protein folding, macromolecule organization and/or transport. Despite the often-highlighted benefits of nanobody-technology, like any other technique their use should be validated including the effect on protein localization.

Artificial modifying localization of proteins can be used on purpose for targeted interference. Chromobodies targeting endogenous actin-binding proteins, like gelsolin and cortactin, led to a disturbed actin distribution^{24, 32-34} paralleled with a decrease in both the number

Figure 3 | Chromobodies may lead to mislocalization of the targeted protein. (A) HEK293T cells expressing protein-GFP without (- Nanobody) or with (+ Nanobody) the presence of chromobodies (anti-GFP nanobody – mCherry – HRP). After 16 hours of transfection, cells were fixed in 4% PFA. Note the localization of protein-GFP difference with and without the presence of the nanobody. Bar: 10 μ m. **(B)** Quantification of normal versus abnormal localized proteins. Especially connexins are affected by the chromobody. Numbers are normalized to 100%. Own data.

of invadopodia as well as extracellular matrix degradation. These factors are important in cell migration³⁵. Thus, chromobodies-assisted protein modulation allows to study the contribution of specific proteins of biology, including cell migration or some of its consequences, like the delay of metastasis³⁴.

Controlled nanobody activation

Spatiotemporal control of chromobody function is desirable in several assays. To initiate functionalized chromobodies in a controlled manner, chemogenetics or light stimuli can be applied to influence the binding capacity of the nanobody during and after synthesis. Such chemogenetic control employs ligand-modulated antibody fragments (LAMAs): a circular permutated bacterial dihydrofolate reductase (cpDHFR) linked to the nanobody is in such a conformation that it recognizes and binds to the nanobody target. In the presence of cell permeable DHFR inhibitors, the conformation changes precluding the antigen binding site of the nanobody binding the target, and thereby loss of association of nanobody and target.

This process can be reversed to activate the nanobody binding³⁶.

The light-dependent nanobody, termed photobody, uses a genetic photocaged tyrosine variant that results in the inactivation of the antigen-binding site^{37, 38}. The photocaged tyrosine is photo-labile, and upon light induction (365 nm) the antigen-binding properties of the chromobodies are restored. Optobody, a second light-dependent tool, uses a split nanobody with a N-terminal fragment containing CDR1 and CDR2, and a C-terminal fragment containing CDR3 (**Figure 2B**)³⁹. When both fragments are genetically fused to an optical-induced dimerization tool (MagHigh⁴⁰), the complete nanobody folds upon light stimuli and thereby forms the antigen-binding site. However, a generic position to split the nanobody is lacking, and thus for every different nanobody optimization and validation is needed. Overall, the activation of nanobodies using light or chemogenetic stimuli gives spatiotemporal control over the nanobodies, allowing precise subcellular modulation followed by direct readout of the biological consequences on targets studied.

1.2 Purified fluorescent nanobodies delivered for live-cell imaging

Nanobodies can also be generated in cellular systems, subsequently purified and/or modified and then used in bioassays. Typically, these are secreted by mammalian cells or produced in high yields by bacteria⁴¹. After purification, the nanobody can for instance be coupled to chemical dyes⁴¹ to create fluorescent nanobodies (**Table 1; 2**). Dyes suitable for super-resolution microscopy (SRM), i.e. LM beyond the diffraction limit resulting in typically 20 – 100 nm lateral resolution (reviewed in⁴²), will increase the resolution when using nanobodies compared to IgGs because of the smaller size of the reagents used, reducing the linkage error discussed above¹⁸. Alternative to conjugation of purified nanobody with small fluorescent molecules, chromobodies can be expressed and purified. These chromobodies can then be directly used in fluorescent microscopy studies because they contain both the targeting module as well as the fluorescent module (**Figure 1**). The benefit of using cDNA encoding protein modules is the ease to switch target or color with molecular cloning tools. To employ these fluorescent nanobodies in live-cells different delivery mechanisms for extracellular or intracellular targets have been created.

Extracellular targets

The extracellular domain of peripheral membrane proteins in cultured cells is well accessible to ectopic added reagents and therefore straightforward to target in live-cell imaging. Nanobodies that target extracellular receptors may trigger receptor specific endocytosis, which can be important for e.g. drug delivery. Endocytosis can be triggered via binding with the human epidermal growth factor receptor 2 (HER2). Anti-HER2 nanobodies⁴³ coupled to fluorescent, drug containing nanoparticles⁴⁴, were indeed able to trigger endocytosis. After the trigger, uptake and cell viability was visualized to examine the effect of the therapeutic nanoparticles. So, receptor mediated endocytosis can be activated using fluorescent nanobodies to study therapeutic agents coupled to the nanobodies.

Super resolution localization of GFP surface-exposed by cells has been achieved while studying dynamic changes at the plasma membrane: The glycosylphosphatidylinositol (GPI)-anchored GFP reporter was further probed with Alexa Fluor 647-conjugated nanobodies to enable SRM based on the blinking of the Alexa-dye. This resulted in higher resolution imaging

of dynamic changes and detection of protein enrichments in the plasma membrane^{18, 45}. Indirect visualization enables newly displayed proteins at the plasma membrane. Here, all available antigens first are blocked by unconjugated nanobody. Upon exocytosis stimuli, at the plasma membrane, new extracellular exposed antigens can be detected with fluorescent nanobodies⁴⁶. This pulse-chase approach allows dynamic studies, e.g. protein turnover, of receptors and other cell surface proteins.

Intracellular targets

The plasma membrane is a physical barrier for the nanobodies to target intracellular proteins in live-cells. Therefore, custom delivery methods are needed to target endogenous proteins in (living) cells without permeabilizing the plasma membrane. If nanobody expression is not an option because it first needs chemical modification or the concentration should be well-controlled, a purified nanobody may be delivered to cells. Fluorescent nanobodies can enter cells via endocytosis, when they formed non-covalent complexes with oligomers (**Figure 2C**)⁴⁷ or they undergo lipid-based protein transfection^{45, 48}. After endocytosis, the nanobodies need to escape the endosomal system and the formed complexes need to be degraded. However, using this strategy one has to take into account that the efficiency of endosomal escape is low⁴⁹ and the nanobodies in the endosomes are already fluorescent, resulting in localized labeling of the endosomal system.

To prevent cellular uptake via endocytosis, cell-permeable nanobodies were generated by the addition of a cyclic cell-penetrating peptide (cCPP;⁵⁰). cCPPs are arginine-rich peptides that facilitates direct penetration of the plasma membrane to enter directly into the cytoplasm, independent of endocytosis. The efficiency of the labeling is expected to be increased because endosomal escape after endocytic uptake is omitted. Another advantage of using the cell-penetrating peptide is the ability to co-transport recombinant proteins, e.g. GFP, inside the cells, when both are bound to the nanobody. Although the efficiency of this co-transport was low, this cell-permeable nanobody can be further explored to serve as a drug delivery vehicle. A major disadvantage of the cCPPs however, is the strong tendency to accumulate in the nucleolus.

Generating temporary permeable plasma membranes is another approach to artificially deliver cargo inside cells. Different methods to temporary permeabilize the membrane have been developed: (i) Electroporation to deliver nanobodies linked to fluorescent quantum dots (QDs) into cells⁵¹. The QDs were used for single particle visualization of intercellular transport by targeting kinesin motor proteins⁵²; (ii) Artificial plasma membrane channels that allow chromobody delivery into cells can be formed by bacterial Streptolysin O⁵³. Unbound nanobodies are removed during rinsing and the channels are closed upon switching to a recovery medium; (iii) Photoporation in which a laser-induced transfection enables the delivery of the nanobodies intracellular, and when these are fluorescently labeled, these can be directly detected⁵⁴. (iv) Another method to induce temporary damage to the plasma membrane is cell squeezing through the small capillaries of a microfluidic system resulting in fragility of the plasma membrane (**Figure 2D**)⁵⁵. While the cells are squeezed, extracellular proteins can diffuse into the cells. When cells leave the small capillary the plasma membrane recovers to its normal state. Obviously, the effect of any temporary permeabilization approach used to enable nanobodies entrance into cells, is that endogenous molecules

might diffuse out or targets may delocalize.

1.3 General applicable peptide tags

Genetic fusion with FPs cDNA is the widely used technique for protein visualization in living systems, but sometimes smaller peptide tags are preferred. Currently, there are no nanobodies available against common generic peptide tags^{10, 56-58}. Hence, three new small tags have been developed along with their respective targeting nanobodies. (i) The BC2-tag is a 12 amino acid peptide sequence originating from β -catenin^{45, 56}, but the nanobody does not recognize endogenous β -catenin making it fairly specific for the tag only. (ii) The ALFA-tag (13 amino acids) forms an α -helix and is naturally absent in eukaryotes⁵⁹, also making it a specific target for its nanobody, which also counts for the (iii) Pep-Tag (15 amino acids)⁶⁰.

An array of peptides fused to the protein of interest can amplify the fluorescent signal, and thus increase the signal-to-noise ratio. A peptide-repeat called MoonTag⁶¹ was created to visualize active translation (**Figure 2E**). Here, an array of a 15 amino acid peptide sequence was added N-terminal of the protein of interest. The newly formed peptide chain forms a docking site for chromobodies. The MoonTag can be combined with the SunTag; an intracellular expressed single-chain variable fragment (scFV;⁶²). Combining the two tags will allow visualization of different reading frames within a single mRNA or can be used to amplify the signal from different targets. Given the mechanism of probing the target with a cDNA encoded peptide repeat, making use of the same antibodies, this is a highly versatile enhancer system.

2. Nanobodies in electron microscopy

While resolution of targets in LM is improved by using nanobodies because of a reduced linkage error compared to IgG targeting, the ultrastructural remains unexplored. In electron microscopy (EM), the ultrastructure is revealed, but localizing the protein of interest within this structure also requires probes. EM-visualization of targets benefits from the nanobody-technology because the probe is small and thus penetrates better. Therefore, milder permeabilization is needed, better preserving the ultrastructure. Moreover, the small size improves the resolution compared to traditional immuno-EM because the target and identifiable tag are in close proximity. Also in EM proteins can be specifically identified using genetically encoded or affinity-based probes. Genetically-encoded probes may be based on peroxidases that creates black precipitates in the presence of diaminobenzidine (DAB) and H_2O_2 . Affinity-based probes include electron dense nanoparticles like nanogold and QDs⁶³. Of course the genetically-encoded probes form good candidates to use in conjunction with nanobodies as a multi-modular probe for EM studies.

2.1 Intracellular nanobody expression

Intracellular nanobodies fused with soybean ascorbate peroxidase (APEX2)⁶⁴ can target GFP or mCherry to add an electron dense mark to the protein of interest at EM level^{65, 66}. This APEX2-nanobody can be applied as general CLEM (correlated LM/EM) probe because many cells and small organisms have already been engineered to express GFP or mCherry. Using conditionally stable nanobodies (section 1), unbound APEX2-nanobody is degraded and thereby improve EM detection of the target proteins⁶⁷. The conditionally stable APEX2-

nanobody enables studying protein-protein interactions by making use of splitFPs as target (**Figure 4A**)^{68, 69}. Here, a protein of interest and its potential interacting protein are genetically fused with different segment of e.g. GFP. Anti-GFP APEX2-nanobody can only bind to the folded GFP, representing the interaction between the two proteins of interest. When GFP is absent, the conditionally stable APEX2-nanobodies are degraded. Another alternative to study protein-protein interactions would be by using nanobodies fused to splitHRP or splitAPEX2^{70, 71}. Two distinct nanobodies recognizing proteins within close proximity will facilitate the refolding of the peroxidase, which will result in DAB precipitates at that location in the sample. The split peroxidase thus reduces the signal of unbound nanobodies, leading to a more conclusive picture. In conclusion, where chromobodies allow dynamic studies of endogenous proteins, APEX2-nanobodies enable high resolution analysis in the ultrastructural context.

Nanobodies as immunolabeling reagent

Purified nanobodies can be applied for affinity-based immunolabeling, and thereby replace conventional IgGs. Immunolabeling with unconjugated nanobodies was used to label HER2 in breast cancer cells, followed by a secondary anti-nanobody IgG conjugated with nanogold (**Figure 4B**)⁷². Although this already improved the resolution, it could benefit more by replacing the IgG a direct labeling method using conjugated nanobodies.

2.2 CLEM and nanobodies

Reagents used in CLEM that are readily detectable both in the FLM and EM, like nanogold or QDs, are relatively large and/ or bulky in comparison to 15-30 kDa range fluorescent proteins or even smaller fluorescent dyes like FITC. These inorganic nanoparticles whether or not conjugated to nanobodies, will have limited penetration^{4, 73}. Therefore, we developed a completely protein-based probe, called “FLIPPER”-body^{19, 74} with (i) a nanobody as targeting module; (ii) a FP for LM analysis; and (iii) a horseradish peroxidase (HRP) for EM analysis. The FLIPPER-bodies were produced by mammalian cells applied as immunoreagent to label e.g. intracellular GFP (**Figure 4C**). Other targets were generated by simple molecular cloning interchanging the modules of the probe. FLIPPER-bodies improve penetration due to its size and flexibility compared to IgGs-targeted nanoparticles and thus lead to a better target detection while maintaining reasonable ultrastructure.

Figure 4 | Nanobody technology for EM. (On the next page) (A) Cells were expressing only C-terminus YFP or both C- and N-terminus YFP. The cells also expressed a conditionally stable anti-YFP, for proteasomal degradation of unbound probe. The nanobodies were genetically fused with peroxidase, APEX2, for EM detection. Note that only black precipitation is visible in cells expressing C-terminus YFP and N-terminus YFP. This confirmed the degradation of the nanobody with the peroxidase. Bars: 1 μ m. (B) Purified nanobodies were used as primary antigen binding protein to reduce the distance between label and antigen. Nanobodies can be detected using an anti-VhH IgG conjugated to gold for EM visualization. Bars: 0.5 μ m. (C) Cells express H2B-GFP from Figure 1A, and are permeabilized after fixation followed by labeling with anti-GFP FLIPPER-bodies. Note the colocalization in LM and the dark, positive nucleus in EM versus an unlabeled nucleus. Bars: 10 μ m. (D) Neuronal cells expressing pHluorin on the plasma membrane. Added anti-GFP nanobodies bind to the pHluorin, and after a pulse stimulation, synaptic vesicles are formed. In EM, a population of unlabeled and labeled synaptic vesicles is detected. Arrows indicate synaptic vesicles, arrow heads indicate unstained vesicles and open arrow heads indicate residual staining (PM: plasma membrane, SV: Synaptic vesicle). Bar: 0.5 μ m. The images in (A-D) have been reproduced from the following studies^{19, 67, 72, 75}.

In conventional EM glutaraldehyde is used to fix the samples for ultrastructural preservation⁴. However, cells fixed with paraformaldehyde become permeable for small molecules like e.g. small fluorescent nanobodies¹⁷. This labeling without adding permeabilization reagents method was further developed to stain intracellular targets in fixed mouse tissue slices. When targeting an intracellular target with both nanobody and IgG, the nanobody penetrated up to 100 μm into a brain tissue slice, while the IgGs only stained the surface of the slice. Subsequently, the same cells were imaged with EM and ultrastructural details were well preserved due to glutaraldehyde fixation after performing the nanobody labeling. So, detergent treatment is not required when using small nanobody probes and thus cellular structure is better preserved. The technique needs to mature further to establish the ratio of positives and false negatives to determine if the recognition of the targets is generically reliable. Moreover, the accessibility of targets within organelles, like mitochondria or nucleus, remains to be studied further.

Nanobodies for live-cell imaging and EM

Alternatively, to expressing nanobody-based probes in the cellular system of interest, they also can first be purified and then applied to cells. An elegant example of this approach has been used to visualize the formation of synaptic vesicles in living cells⁷⁵. Ultrastructural localization of newly formed synaptic vesicles can be examined using peroxidase-fused nanobodies. Vesicle-associated membrane protein 2 (VAMP2) mediates the formation of synaptic vesicles and is expressed at the plasma membrane of neuronal cells. Anti-GFP nanobodies were applied to target pHluorin, a GFP derivative, fused with VAMP2 (**Figure 4D**). Upon K^+ stimulation, pHluorin-VAMP2 with bound nanobody is endocytosed, and new synaptic vesicles are formed. After EM preparation, the newly formed synaptic vesicles were stained black, as a result of the peroxidase attached to the nanobody, whereas older synaptic vesicles remained unstained. Thus, the endocytic route of specific proteins of interest can be visualized.

Purified nanobodies, fused to a FP and a peroxidase, were used to analyze the retrograde transport system in live-cells⁷⁶ or within ultrastructural context⁷⁷ to study the transport from the cell surface to the Golgi complex. Cells with GFP-modified cycling reporter proteins at the plasma membrane captured the nanobodies extracellular and transported them in the cells. CLEM revealed the dynamic behavior of different cycling reporter proteins, and showed the ultrastructural localization of the reporter proteins. Overall, these nanobody-based CLEM probes can reveal the localization of plasma membrane proteins and visualize their dynamics, together with the ultrastructural context.

2.3 Nanobodies in cryo-EM

Structural analysis of proteins at atomic resolution is achieved by cryo-EM (reviewed in⁷⁸⁻⁸⁰). However different conformations of proteins may be present, hindering the structural determination of specific states. Here, nanobodies can help to stabilize the proteins into a certain conformational state (reviewed in⁸¹), especially as the targeting module in rigid chimeras termed megabodies. These stabilizing nanobody-chimeras recently have been used to solve the type A γ -aminobutyric (GABA_A) structure in membranes-like structures in presence and absence of natural ligands and antagonists^{82, 83}. Using the megabodies, no longer the target proteins themselves need to be engineered and thus structure on the

endogenous proteins is being revealed. Likely, nanobody technology will further contribute to structural biology on many other target proteins by facilitating single conformational protein states and better understand the dynamic regulation of biomolecules by their ligands without directly altering the proteins at study.

3. Nanobodies for microscopy: To date a great potential but such a limited use

Nanobodies have clear benefits over conventional IgGs antibodies or genetically encoded probes. Nanobodies (i) have a small diameter resulting in better resolution and better penetration; (ii) can visualize endogenous proteins in live-cell imaging; (iii) are encoded by a one cDNA, which enables easy molecular cloning; (iv) allow researchers to create and produce custom multi-modal probes. Potential artefacts specific for nanobody technology in microscopy include signal from chromobodies independent of target binding and modified localization of the protein target, as detailed above.

Nanobodies are still limited used in research, mainly due to the limitation in the availability of nanobodies for general targets compared to IgGs and researchers are not aware of the possibilities of nanobody in microscopy. Here, we aim to increase the visibility of opportunities and benefits highlighted in the main text, but also exemplified by the successful new insights and new nanobody-based reagents by many (**Table 2**). Other ways to improve the use of nanobodies in research are open sharing of nanobodies cDNA to allow researchers to create and manipulate their own labeling reagent and to increase the nanobody database of general targets. Currently, most nanobodies are generated via Camelidae immunization, a route that may be a (seemingly) hurdle for scientists. However, these days the generation of nanobodies is both economically and time-wise competitive with generation of newly synthesized rabbit IgG against specific targets. The novice user obviously will benefit of expertise and accessibility to the needed infrastructure by more experienced users mentioned throughout this review. In addition, a fast method based on a yeast display platform to select nanobodies in vitro has been established¹⁶.

4. From current state to future outlook: Nanobodies towards a common technique

Protein identification in microscopy has been greatly aided by immunolabeling using IgGs as well as the application of genetically-encoded tags. Nanobody technology, being explored for approximately 25 years is a great additional tool. Like IgGs, endogenous proteins can be easily studied without direct modification. Moreover, the single domain properties and therefore the easy use as a module in other genetically-encoded probes allow freedom of use of tags and targeting module. In line with SRM techniques, nanobodies have the added benefit over IgGs that they hardly provide distance between target and label (linkage error). The major limitation in the use of nanobodies in general is the sparse availability of high affinity nanobodies for specific targets. Increasing the availability for different targets using immunization and/ or micro-organism-based libraries will increase the variation in the decade to come. When more targets can be studied with nanobodies these will become a common tool in the lab and share the top-3 podium with genetically-encoded tags as well as IgGs since they share benefits of both approaches to identify and visualize targets of interest in dynamic systems and with high precision.

Author contribution

MAdB performed the experiment and made the figures. MAdB and BNGG wrote the manuscript.

Acknowledgement

We thank our lab members for discussions; financial support by The Netherlands Organization for scientific research (STW Microscopy Valley 12718, NWO 175-010-2009-023 and ZonMW 91111.006) and de Cock-Hadders Stichting.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Chapter 3a

A small protein probe for correlated microscopy of endogenous proteins

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Histochemistry and Cell Biology (2018) 149(3):261-268
doi: 10.1007/s00418-018-1632-6

Abstract

Probes are essential to visualize proteins in their cellular environment, both using light microscopy as well as electron microscopy (EM). Correlated light microscopy and electron microscopy (CLEM) requires probes that can be imaged simultaneously by both optical and electron dense signals. Existing combinatorial probes often have impaired efficiency, need ectopic expression as a fusion protein or do not target endogenous proteins. Here we present FLIPPER-bodies to label endogenous proteins for CLEM. FLIPPER (fluorescent indicator and peroxidase for precipitation with EM resolution), the combination of a fluorescent protein and a peroxidase, is fused to a nanobody against a target of interest. The modular nature of these probes allows an easy exchange of components to change its target or color. A general FLIPPER-body targeting GFP highlights histone2B-GFP both in fluorescence and EM. Similarly, endogenous EGF receptors and HER2 are visualized at nm-scale resolution in ultrastructural context. The small and flexible FLIPPER-body outperforms IgG-based immunolabeling, likely by better reaching the epitopes. Given the modular domains and possibilities of nanobody generation for other targets, FLIPPER-bodies have high potential to become a universal tool to identify proteins in immuno-CLEM with increased sensitivity compared to current approaches.

Keywords: Probes, Correlated microscopy, FLIPPER, Nanobody, APEX2

Introduction

An ultimate goal in microscopy is to detect targets at both the fluorescent level, opening the possibilities to study live cells and investigate large areas, as well as detection using electron microscopy (EM) to allow localization with molecular precision (nanometer range) in the context of ultrastructure. These benefits are all combined in correlated light microscopy (LM) and EM (CLEM; reviewed in^{1, 2}). In CLEM probes, both the fluorescent and electron dense signal need to be specifically targeted to the protein of interest.

Specific probe targeting for endogenous proteins is usually done with antibodies, which can be applied before (pre-embedding) or after EM preparation (post-embedding). The advantage of post-embedding labeling is the preserved ultra-structure, but antigen binding is impaired due to EM preparation. Pre-embedding labeling has a compromised ultrastructure partly as a result of the permeabilization, but a good accessibility to the antigens^{1, 3}. Another disadvantage of antibodies is their size: 150 kDa with a length of 14 nm for typical IgG (**Figure 1A**). This size results in a distance up to 28 nm between target and label^{4, 5}.

Nanobodies-mediated targeting reduces the distance of target and probe. Nanobodies are the smallest antigen binding proteins that are derived from heavy-chain antibodies of Camelidae species, consisting of a single chain, as opposed to IgGs^{9, 10}. Genetic fusion of nanobodies with fluorescent proteins (FPs; chromobodies¹¹), as well as with ascorbate peroxidase 2 (APEX2) for EM visualization¹² have been used in co-expression systems and successfully show identification of several targets. However, co-expression in cells also affects protein function for some target¹⁰; see below). Indirect EM-labeling using an anti-nanobody antibody and a protein-A conjugated to gold particles was recently pioneered,

Figure 1 | Small FLIPPER-bodies for single step CLEM labeling. (A) Left: Traditional indirect antibody labeling conjugated with QD, represented by black dot. Right: A single labeling step with FLIPPER-body. Black circle represents DAB conversion. Black triangle: antigen. Bar: 2nm. Adapted from⁶⁻⁸ **(B)** Boxes representing the modules of the anti-GFP and anti-EGFR FLIPPER-bodies. The boxes are shown in proportion to each other. Signal peptide (#), His6-tag (*), thrombin cleavage site (^), mCherry (mCh), dTomato (dTom), horseradish peroxidase (HRP), ascorbate peroxidase 2 (APEX2), anti-GFP nanobody (antiGFP), anti-EGFR nanobody (antiEGFR).

but still results in a relatively large distance, >17 nm, from the gold particle to the target protein (HER2)¹³. We reasoned that a single multi-domain protein that (1) can specifically target proteins based on a nanobody, (2) directly visualizes these using fluorescent proteins, and (3) identifies targets in EM using peroxidase-mediated DAB-precipitation may have better labeling efficiency than particle-based detection in EM. Since the architecture of the genetically-encoded probes allows easy domain swapping, virtually unlimited combinations between targeting modules, FPs and peroxidase can be made (**Figure 1B**). Note that, as opposed to most current genetically-encoded probes, our FLIPPER-bodies are first secreted by producer cells and subsequently used, either as a supernatant or purified, like hybridoma generated monoclonal antibodies. Our results show that this protein-based probe highly efficiently labels targets, including in difficult to reach subcellular areas, and outperforms current available alternatives.

Results

Previously, we introduced the combination of FP and HRP linked to a protein of interest as a probe for CLEM inside cells, named FLIPPER¹⁴. In addition, APEX2¹⁵ is used to generate the EM-detectable signal. FLIPPER-bodies are multi-modular fusions between a nanobody, a FP and a peroxidase (**Figure 1B**). Because of the inclusion of a signal peptide, the FLIPPER-body is secreted by producer CHO cells and the conditioned media is used to label other cells. Alternatively, using the included His-tag the FLIPPER-bodies can be purified. The different FLIPPER-bodies below were all produced by mammalian cells. As a first test for expression and activity, the medium was simply tested for DAB conversion (**Figure S1**).

FLIPPER-bodies visualize nucleus-localized GFP at the LM and EM levels

As a proof-of-concept a FLIPPER-body was created consisting of a nanobody targeted to GFP fused to mCherry and HRP. After fixation and permeabilization H2B-GFP positive cells were labeled directly with an anti-GFP FLIPPER-body only or as a control an antiGFP IgG that was detected with a secondary anti-body conjugated to quantum dot 655 (QD655), another CLEM probe for immunolabeling (**Figure 2**)^{16,17}. H2B-GFP expression is clearly visible in the nucleus, while the limited penetration of the QDs can be seen by detection of some QDs in the cytoplasm and not in the nucleus (**Figure 2A**). None of the other secondary antibodies conjugated to either Alexa594, HRP or 10-nm gold particles could be discerned in the nucleus either (**Figure S2**). In contrast, the FLIPPER-body labeling shows in GFP positive nuclei both a mCherry signal and the electron-dense signal derived from DAB-conversion (**Figure 2B**). Note that the signal is only found in the H2B-GFP transfected cells only. These results demonstrate the functionality of FLIPPER-bodies for intranuclear labeling of proteins, as well as the feasibility to target GFP. Therefore, we set out if we can use FLIPPER-bodies as generic tools, starting with targeting a cell surface receptor.

Specific labeling of the EGFR by FLIPPER-bodies

EGFR was chosen as the second target to optimize and verify FLIPPER-body technology. The new probe, consisting of a well-characterized anti-EGFR nanobody (7D12)¹⁸ was positioned at the N-terminal of the FP and APEX2 modules (**Figure 1B**). We switched the order because binding capacity of the nanobody was lost after C-terminal conjugation. dTomato was chosen as FP, because its dimer-forming capacity that may double the fluorescence and EM signals.

Figure 2 | FLIPPER-body labeling of nuclear localized GFP. HEK293T cells expressing H2B-GFP. (A) H2B-GFP was detected by indirect labeling with rabbit anti-GFP and secondary IgG conjugated to QD655. Note that fluorescence detection of QDs is limited to the cytoplasm, which may be non-specific labeling. High resolution EM images also show only QDs in the cytoplasm. **(B)** H2B-GFP was detected by direct labeling with FLIPPER-bodies containing mCherry. The used FLIPPER-body is shown at the right. Note the specificity of the mCherry signal in only the GFP positive cells. With EM the black DAB precipitates are seen in the nuclei. DIC: differential interference contrast; GFP: GFP fluorescence; QD655: QD655 fluorescence; mCherry: FLIPPER-body; merged: GFP and mCherry; EM: ultrathin EM section. Bars: LM and EM 10 μ m, EM zoom in 1 μ m. Unbiased large-scale high resolution EM images are available via nanotom.org.

EGFR-GFP overexpressing cells were targeted with traditional immuno-CLEM using IgGs and QDs as described above, as well as with anti-EGFR FLIPPER-body by pre-embedding labeling using non-permeabilized cells. The EGFR-GFP signal is present in the Golgi system as well as on the plasma membrane of the cells (**Figure 3**). Traditional labeling clearly revealed QD fluorescence at the apical plasma membrane, but significantly less at cell-cell contact sites (**Figure 3A**). Secondary antibodies conjugated with other labels showed similar results (**Figure S3**). FLIPPER-body mediated labeling of EGFR was clearly visible by the dTomato signal as well as by the electron dense staining (**Figure 3B**). Note that the signal was distributed evenly over the surface of the cells, including at sites of cell-cell contacts that were not visualized with the traditional methods.

3a

Figure 3 | FLIPPER-body targets specific overexpressed EGFR. HEK293T expressing EGFR-GFP **(A)** Detection of EGFR by GFP fluorescence, as well as by indirect labeling of EGFR with secondary antibodies conjugated to QD655. Note that QD655 are detected at the periphery of the cells, but not at cell-cell contact sites **(B)**. Detection of EGFR using FLIPPER-body (cartoon right). The FLIPPER-body labels uniquely the EGFR-GFP positive cells, including at the cell-cell contact sites. DIC: differential interference contrast; GFP: GFP fluorescence; QD655: QD655 fluorescence; dTomato: FLIPPER-body; merged: GFP and label; EM. Bars: LM 10 μ m, EM 5 μ m, EM zoom in 0.5 μ m. High resolution EM images are available via nanotomoy.org.

Figure 4 | FLIPPER-body reveals localization of endogenous EGFR. (A) EGFR on A431 cell was labeled using anti-EGFR and secondary IgG conjugated with QD655. Note that the QD655 labeling is mainly restricted to the periphery of the cell clusters. **(B)** EGFR is labeled with FLIPPER-body shown at the right. The cells periphery as well as the cell-cell contact sites are labeled with fluorescence and black staining in EM. DIC: differential interference contrast; QD655: QD655 fluorescence; dTomato: FLIPPER-body. Bars: LM 20 μ m, EM 10 μ m and EM zoom in 200 nm. High resolution large-scale EM images are available via nanotomography.org.

Endogenous detection of EGFR

Next we used A431 cells, known for its high EGFR levels¹⁹, to investigate our FLIPPER-body technology for detection of endogenous proteins. The A431 cells were fixed and labeled with the different probes. Indirect labeling of EGFR using QDs resulted in a labeling of the periphery of the cell clusters while the cell-cell contacts were devoid of any labeling (**Figure 4A**). The decreased labeling efficiency at cell-cell contact sites was also seen using IgGs with

other labels, even with the small moiety Alexa546 (**Figure S4**). Labeling efficiency of the EGFR was much higher with anti-EGFR FLIPPER-body (**Figure 4B**). Not only the periphery of the cell clusters were labeled with high efficiency using as assessed by dTomato fluorescence and electron dense precipitates for LM and EM respectively, but also the cell-cell contacts sites also have efficient labeling.

A third target: HER2 detection with correlated microscopy

FLIPPER-bodies targeting HER2 were created by switching the anti-EGFR nanobody for an anti-HER2 nanobody (11A4). The HER2 nanobody was well characterized in binding capacity and specificity²⁰. HER2 is known for its high expression level in breast cancer²¹, therefore the breast cancer cell line SkBr3 was labeled. HER2 detection with FLIPPER-bodies shows high label efficiency in both modalities (**Figure 5A**), as emphasized in the CLEM overlay (**Figure 5B**). Between the cell-cell contacts labels are detected (**Figure 5C**), suggesting the good penetration of this FLIPPER-body. Some cells have regions with less label efficiency in fluorescence as well as in electron density (**Figure 5C**). This suggests an irregular distribution of HER2 in the plasma membrane, which is in agreement with previous reports²².

Figure 5 | Correlated microscopy of endogenous HER2. SkBr3 cells labeled and processed for CLEM. (A) HER2 detection with anti-HER2 FLIPPER-body with dTomato fluorescence. In EM, a black substrate is surrounding the cells. **(B)** overlap of the fluorescence and EM image resulting in the CLEM image. **(C)** Zoom in EM images shows the labeling at the cells periphery and cell-cell contact sites (green zoom in). It is also noted that there is a heterogeneous distribution of the HER2 labeling (blue zoom in). DIC: differential interference contrast; LM: dTomato fluorescence; EM: ultrathin EM section. Bars: LM 10 μ m, EM 10 μ m and EM zoom in 500 nm. High resolution EM images are available via nanotomy.org.

Discussion

Genetically-encoded FLIPPER-bodies were created as an alternative for multiplex immunolabeling with nanoparticles for CLEM. Proof-of-concepts that the single step nanobody-based labeling is more efficient and penetrates better than traditional immunolabeling using IgGs is given in examples of nuclear labeling as well as for endogenous plasma membrane receptor labeling, including in cell-cell contact sites. Importantly, this experimental result is a better reflection of receptor localization that would be missed by traditional CLEM or EM antibody-based labeling strategies, which does not label nuclear proteins or receptors at cell-cell contacts (see results above and Schnell et al., 2012)³. Improved labeling with FLIPPER-bodies may be caused by (i) better penetration into dense structures as a result of their small size (~75 kDa) as compared to the size of IgG antibodies (~150 kDa) that are linked to nanoparticles (**Figure 1A**); (ii) The FLIPPER-body requires a single labeling step, the signal is not enhanced by amplification in a second step, but all target-reaching FLIPPER-bodies will have label; (iii) FLIPPER-bodies are small proteins, so it is a flexible molecule which might more easily reach the epitope in contrast to QDs or gold particles¹⁶. Even compared to the bulky IgGs conjugated to a couple of small Alexa dyes, FLIPPER-bodies can reach the epitope better.

The FLIPPER-body technology represents a versatile system that can be designed to bind to any possible target, and can be provided with any possible color or peroxidase by simple molecular cloning. FLIPPER-bodies are preferentially as small as possible and easily produced and secreted in a production cell line like CHO cells. As nanobodies are single chain binding units, small, and to be generated against any possible target, the nanobody is currently the preferred binding unit. Moreover, given the versatility of nanobody applications⁸ and recent establishment of a centralized nanobody database collection (ICAN)²³, the expansion of the FLIPPER-bodies has great promise. However, alternative small, single domain binding scaffolds capable of binding with high affinity to their target such as affibodies may also be employed in the FLIPPER-body technology¹⁰. Note that because of the genetic encoding, molecular biology tools easily will help to refine the FLIPPER-bodies, like the continuous improvements we have seen for fluorescent proteins and genetically-encoded biosensors^{6, 24}. Also note that, like all plasmids or hybridomas, the FLIPPER-bodies can be shared free of charge within the scientific community, as opposed to antibodies. Our future studies will assess whether the FLIPPER-bodies can also be used in Tokuyasu-like immuno-EM²⁵ or Epon-section labeling²⁶ and might also be a generic tool for post-embedding labeling.

A limitation of peroxidase based probes, like the FLIPPER-body is that in EM, labeling multiple targets cannot be distinguished, while with QDs, it is possible to distinguish multiple targets due to differences in shape¹⁶. One way of using multiple targets would be using multiple fluorescent colors and later layering the images to see where each target is localized. Another option for double labeling makes use of DAB with specific incorporated lanthanides detectable with an electron energy detector²⁷. In this case, after the first labeling Cerium-DAB₂ with is added and blocked, followed by the second label and black precipitates are formed with Lanthanum-DAB₂. The different lanthanides then can be distinguished. Similarly, X-ray detection may possibly be used to discriminate between certain La-containing DAB precipitates, which may be even multiplexed with gold particles or QDs²⁸.

In conclusion, FLIPPER-bodies have the potential to become a generic probe to identify proteins in CLEM with significant benefits over existing immuno-EM using IgGs, most importantly in access to the epitope.

Acknowledgment

We acknowledge Klaas Sjollemma for technical assistance and all our lab members for feedback and discussion; financial support by The Netherlands organization for scientific research (NWO 175-010-2009-023; ZonMW 91111.006; STW Microscopy Valley 12718) and Jan Kornelis de Cock Stichting.

Conflict of Interest

The authors declare they have no conflict of interest.

Author Contributions

BNGG conceived the idea and created the website. MAdB performed all experiments. BNGG & MAdB wrote the manuscript. JK and PMPBH advised and supplied reagents and/or training. All authors analyzed data and commented on the manuscript.

Material and Methods

Plasmids

The complete cDNA sequences of the used FLIPPER-bodies are presented (Supplementary data). The backbones of the FLIPPER-bodies were ordered by Eurofins Genomics (Ebersberg, Germany) and modules were switched with standard molecular cloning tools. The signal peptide originated from EpCAM. The cDNA of mCherry and dTomato (gifts from Roger Tsien)²⁹ were inserted using *BamHI* and *EcoRI*. Horseradish peroxidase (HRP) was replaced by APEX2 (gift from Alice Ting: Addgene plasmid # 49385)¹⁵ using *EcoRI* and *NotI*. Anti-EGFR (epidermal growth factor receptor) nanobody was replaced by anti-HER2 (Human Epidermal growth factor Receptor 2) using *AgeI* and *EcoRI*. Other plasmids used were EGFR-GFP (gift from Alexander Sorkin: Addgene plasmid # 32751)³⁰, Histone2B (H2B)-GFP (gift from Geoff Wahl: Addgene plasmid # 11680)³¹ and SP-His6-GPI-CFP³².

Cell culture and transfection

HEK293T, A431 and SkBr3 cells were cultured in DMEM (Invitrogen), and CHO-K1 cells were cultured in DMEM/Ham's F-12 1:1 (Lonza), all supplemented with 5% Fetal Bovine Serum (FBS, Greiner) and penicillin/ streptomycin (PAA) at 37 °C in the presence of 5 % CO₂. Cells were transfected using 1:4 DNA/polyethylenimine 1 mg/ml (PEI MW 25,000, Polyscience). Stable CHO-K1 cell lines producing anti-GFP and anti-EGFR FLIPPER-bodies were selected using 1 mg/ml G418 (Invitrogen) and subsequent FACS-sorted (MoFlo Astrios, Central Flowcytometry Unit, UMC). For imaging HEK293T and A431 cells were grown on glass bottom dishes (Greiner); SkBr3 cells were grown on coordinated glass bottom dishes (MatTek).

Fixation

The cells were fixed by adding drop wise an equal volume of 2% paraformaldehyde/0.2 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 to the medium. After 10 min, the medium/fixative was replaced by the fixative described above for 30 min and rinsed once 5 min with 0.1 M sodium cacodylate buffer and twice 5 min with Phosphate Buffered Saline (PBS) pH 7.3. HEK293T expressing H2B-GFP were permeabilized with 0.1 % Triton X-100 in PBS for 7 min and washed three times 5 min with PBS. All steps were performed at R.T.

FLIPPER-body production

FLIPPER-bodies are produced and secreted by stable CHO cells or by transiently transfected HEK293T cells. Conditioned media was collected after two days of culturing. The activity of the secreted peroxidase was tested as a 1:1 mixture of medium and diaminobenzidine (DAB, 5 mg DAB in 10 ml PBS, filtered through Whatman 0.2 µm filter with an additional 3 µl of 30% H₂O₂).

Antibodies

Primary antibodies used were rabbit anti-GFP (Rockland, 600-401-215, 1:200) and mouse anti-EGFR (Santa Cruz, sc120, 1:200). Secondary antibodies used were goat anti-mouse Alexa Fluor 546 (Invitrogen, A11030, 1:500), donkey anti-rabbit Alexa Fluor 594 (Invitrogen, A21207, 1:500), goat anti-mouse QD655 (Life Technologies, Q11021MP, 1:500), goat anti-

rabbit QD655 (Life technologies, Q11421MP, 1:500), sheep anti-mouse HRP (GE-Healthcare, NA931V, 1:500), donkey anti-rabbit HRP (GE-Healthcare, NA934V, 1:500), goat anti-mouse 10 nm gold (BBI solutions, EM.GMHL10, 1:50), goat anti-rabbit 10 nm gold (BBI solutions, EM.GAR10, 1:50).

Immunolabeling, confocal microscopy

HEK293T, A431 and SkBr3 cells were labeled with FLIPPER-bodies for 45 min and rinsed twice 5 min with PBS. Primary and secondary antibodies were diluted in 1 % BSA/PBS and followed by rinsing three times with PBS. Next, the cells were incubated with Hoechst (Sigma-Aldrich, 861405, 1 μ g/ml), for 10 min, except H2B-GFP labeled with QDs, and rinsed three times 5 min with PBS. All steps were performed at room temperature. Fluorescent images were generated using confocal imaging (Zeiss LSM 780, Plan-Neofluar 63 $\times$ /N.A. 1.3 Imm Korr DIC M27 lens).

DAB polymerization, embedding, electron microscopy

DAB (5 mg DAB in 10 ml PBS, filtered through Whatman 0.2 μ m filter with an additional 3 μ l of 30% H₂O₂) was added to the cells for 15 min. Afterward the cells were rinsed twice for 5 min with PBS. Samples were prepared for EM as described previously¹⁴. In short, the cells were post-fixed with 1% OsO₄ in 0.1 M sodium cacodylate buffer and dehydrated with an increasing ethanol series. Finally, the cells were embedded in epon. The glass bottom was removed using hydrogen fluoride for 10 min.

Ultrathin sections (80nm) were obtained and collected at 150 mesh copper grids. Sections were not contrasted with uranyl or lead. The samples were pre-irradiated using FEI CM100 TEM to stabilize them in the electron beam and images were recorded using a Zeiss Supra55 scanning microscope^{26, 33}. STEM images were taken with 2.5 nm pixel size at 28 KV. All data are available at www.nanotomy.org.

CLEM workflow

For all experiments, cells were fixed and labeled, either with or without permeabilization. Fluorescence microscopy was performed at the wet, fixed samples. Subsequently, the DAB-precipitates were formed and cells were processed for EM, using osmium fixation and epon embedding. While this procedure leads to loss of fluorescence, reviewed in de Boer et al. (2015)¹, the FLIPPER-bodies allow identification of the targets at the nanometer-range scale by analysis of osmiophilic DAB-polymers. For a more complete flow-chart of alternative labeling procures see de Boer et al. (2015)¹.

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Supplementary information

Figure S1 | Secreted FLIPPER-body contains active peroxidase. Medium of CHO-K1 cells expressing SP-His6-GPI-CFP (left) or FLIPPER-bodies (right) was analyzed for the presence peroxidase activity. Note that following DAB conversion, His6-GPI-CFP do not react with DAB and the FLIPPER-bodies do provide a DAB reaction.

3a

Figure S2 | Detection of nuclear GFP shows limited penetration of IgG – based labeling. HEK293T cells transiently expressing H2B-GFP were fixed, permeabilized and indirectly stained with anti-GFP. **(A)** Secondary antibody conjugated to Alexa Fluor594 were used to detect nuclear expressed H2B-GFP and while there is clear GFP signal in the nucleus, the A594 signal is absent from the core of the nucleus. There is also much background of the A594 in the cytoplasm. **(B)** Secondary antibodies conjugated to HRP were used to detect nuclear expressed H2B-GFP. Both at low as well as at high EM resolution no signal, representing HRP after DAB conversion could be detected. **(C)** Secondary antibodies bound to 10nm gold label were used to detect nuclear H2B-GFP. Some background signal was observed in the cytoplasm, which can be seen in the insert. **(D)** FLIPPER-bodies were used to detect nuclear H2B-GFP. Note the positive mCherry and HRP signal in LM and EM, respectively. DIC: differential interference contrast; GFP: GFP fluorescence; 594nm: secondary antibody conjugated to Alexa594; mCherry: fluorescence from FLIPPER-body; merged: GFP and indicated fluorescent signals; EM: ultrathin EM section. Bars: LM and EM overview 10 μ m, EM zoom in 1 μ m. High resolution EM images are available via nanotomy.org.

Figure S3 | Detection of overexpressed EGFR using traditional antibody-based probes. HEK293T cells were transiently transfected with EGFR-GFP cDNA, fixed, pre-embedding labeled and processed for LM and EM. **(A)** Indirect labeling using anti-EGFR and secondary antibodies conjugated to Alexa Fluor 546 was used for detection of EGFR, showing an efficient label surrounding the cells. **(B)** Indirect EGFR labeling using HRP-conjugated antibodies shows an efficient labeling at the cell clusters periphery. The zoom in in EM shows the limited penetration between cell-cell contact sites. **(C)** EGFR detection using secondary antibodies bound to 10nm gold seen in the EM magnification. Gold particles decorate the cells periphery, but not at cell-cell contacts sites. **(D)** Direct labeling of EGFR using FLIPPER-bodies showing in LM and EM high label efficiency at the cells periphery and at cell-cell contact sites. DIC: differential interference contrast; GFP: GFP fluorescence; 546nm: secondary antibody conjugated to Alexa546; dTomato: fluorescence from FLIPPER-body; merged: GFP and indicated fluorescent signals; EM: ultrathin EM section. Bars: LM 10 μ m, EM 500 nm, Zoom in 100 nm. High resolution EM images are available via nanotomy.org.

3a

Figure S4 | Detection of endogenous EGFR using different traditional labeling methods. A431 cells are fixed and labeled with anti-EGFR antibodies, detected with common used secondary antibodies conjugated to **(A)** Alexa546, **(B)**, HRP, or **(C)**, 10 nm gold particles. All cells have labeling outside the cell clusters but lack in the detection of EGFR between the cell contacts. **(D)** Direct labeling of EGFR with the FLIPPER-body, labeling all the cells periphery and cell-cell contact sites. DIC: differential interference contrast; 546nm: secondary antibody conjugated to Alexa546; dTomato: fluorescence from FLIPPER-body; EM: ultrathin EM section. Bars: LM 20 μ m, EM overview 10 μ m, zoom in 200 nm.

Supplementary sequence data

- Signal peptide (SP)¹
- 6-Histidine tag (HIS)
- Thrombin cleavage site²
- mCherry (mCh)³
- dTomato (dTom)³
- Horseradish peroxidase (HRP)⁴
- Ascorbate peroxidases 2 (APEX2)⁵
- Nanobody (NB):
 - antiGFP⁶
 - antiEGFR⁷
 - antiHER2⁸

Sequence anti-GFP FLIPPER-body

SP-HIS-Thrombin-mCh-HRP-NB

aagcttcttaaggatatcaccatggcgccccgcaggtcctcgcggttcggggttctgcttgccgcggcgacggcg
M A P P Q V L A F G L L A A A T A
acttttgccgcagctcaggaagaagatatcatgcatcatcatcaccaccatcatcatctcgtaccaagaggttct
T F A A A Q E E D I M H H H H H H H L V P R G S
gttaacgaattcatggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaagggtg
V N E F M V S K G E E D N M A I I K E F M R F K V
cacatggaggggtccgtgaacggccacgagttcgagatcgagggcgagggcgagggcgcccccctacgagggcacc
H M E G S V N G H E F E I E G E G E G R P Y E G T
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Q T A K L K V T K G G P L P F A W D I L S P Q F M
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Y G S K A Y V K H P A D I P D Y L K L S F P E G F
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gagttcatctacaaggtgaagctgcgcggcaccaacttccccctccgacggccccgtaatgcagaagaagaccatg
E F I Y K V K L R G T N F P S D G P V M Q K K T M
ggctgggaggcctcctccgagcggatgtaccccaggacggcgccctgaagggcgagatcaagcagaggctgaag
G W E A S S E R M Y P E D G A L K G E I K Q R L K
ctgaaggacggcgccactacgacgtgaggtcaagaccacctaagccaagaagcccgatgcagctgccccggc
L K D G G H Y D A E V K T T Y K A K K P V Q L P G
gcctacaacgtcaacatcaagttggacatcacctcccacaacaggactacaccatcgatggaacagtacgaacgc
A Y N V N I K L D I T S H N E D Y T I V E Q Y E R
gccgagggcgccactccaccggcgcatggacgagctgtacaaggatccatgcagttaacccctacattctac
A E G R H S T G G M D E L Y K G S M Q L T P T F Y
gacaatagctgtcccaacgtgtccaacatcgttcgacacacaatcgtaacgagctcagatccgatcccaggatc

D N S C P N V S N I V R D T I V N E L R S D P R I
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 A A S I L R L H F H D C F V N G C D A S I L L D N
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 M K A A V E S A C P R T V S C A D L L T I A A Q Q
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 S V T L A G G P S W R V P L G R R D S L Q A F L D
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 L A N A N L P A P F F T L P Q L K D S F R N V G L
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 N R S S D L V A L S G G H T F G K N Q C R F I M D
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 R L Y N F S N T G L P D P T L N T T Y L Q T L R G
 ttgtgccactgaatggcaacctcagtgactagtgactttgatctgcggaccccaaccatcttcgataacaag
 L C P L N G N L S A L V D F D L R T P T I F D N K
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 Y Y V N L E E Q K G L I Q S D Q E L F S S P N A T
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 R M G N I T P L T G T Q G Q I R L N C K V V N S N
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 G R F T I S R D D A R N T V Y L Q M N S L K P E D
 acggccgtgtattactgtaatgtgaacgtgggcttcagatattggggccaaggcaccaggtcaccgtctccagc
 T A V Y Y C N V N V G F E Y W G Q G T Q V T V S S
 taaaccggt

*

Sequence anti-EGFR FLIPPER-body

SP-NB-dTom-APEX2-HIS

gatatcaagcttatggcgccccgcaggtcctcgcttcgggcttctgcttgccgcgggcgacggcgacttttgc
 M A P P Q V L A F G L L L A A A T A T F A
 gcagctcaggaagaaaccggt Nanobody anti-EGFR gaatcatggtgagcaagggcgaggaggtcatcaa
 A A Q E E T G E F M V S K G E E V I K
 gagttcatgcgcttcaagtgcgcatggagggtccatgaacggccacgagttcgagatcgaggcgaggggcgag
 E F M R F K V R M E G S M N G H E F E I E G E G E

ggccgccccctacgagggcaccagaccgccaagctgaaggtgaccaagggcgccccctgcccttcgctgggac
G R P Y E G T Q T A K L K V T K G G P L P F A W D
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I L S P Q F M Y G S K A Y V K H P A D I P D Y K K
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L S F P E G F K W E R V M N F E D G G L V T V T Q
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D S S L Q D G T L I Y K V K M R G T N F P P D G F
gtaatgcagaagaagaccatgggtctgggagggcctccaccgagcgctgtacccccgagcggcgctgctgaagggc
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K P V Q L P G Y Y Y V D T K L D I T S H N E D Y T
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S **G K S Y P T V S A D Y Q D A V E K A K K K L R G**
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F I A E K R C A P L M L R L A F H S A G T F D K G
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T K T G G P F G T I K H P A E L A H S A N N G L D
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I A V R L L E P L K A E F P I L S Y A D F Y Q L A
ggcgttgttgccgttgaggtcacgggtggacctaaaggttccattccaccctggaagagaggacaagcctgagcca
G V V A V E V T G G P K V P F H P G R E D K P E P
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G L L Q L P S D K A L L S D P V F R P L V D K Y A
gcggacgaagatgccttcttctgctgattacgctgagggtcaccaaaagctttccgagcttgggttctgctgatgc
A D E D A F F A D Y A E A H Q K L S E L G F A D A
gcggccgctcatcatcatcaccaccat**taactcgagctctaga**
A A A **H H H H H H** *

3a

Sequence anti-HER2 FLIPPER-body

SP-NB-dTom-**APEX2-HIS**

gatatcaagctt**atg**gcgccccgcaggtcctcgcggttcgggcttctgcttgccgcgggcgacggcgacttttgc
M A P P Q V L A F G L L A A A T A T F A

gcagctcaggaagaaaccggt

A A Q E E T G

Nanobody anti HER2

gaattcatgggtgagcaag

E F M V S K

ggcgaggaggtcatcaaagagttcatgcgcttaagggtgcgcattggagggtccatgaacggccacgagttcgag

G E E V I K E F M R F K V R M E G S M N G H E F E

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ctgcccttcgcctgggacatcctgtccccccagttcatgtacggctccaaggcgtacgtgaagcaccgcccgac

L P F A W D I L S P Q F M Y G S K A Y V K H P A D

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L V T V T Q D S S L Q D G T L I Y K V K M R G T N

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F P P D G P V M Q K K T M G W E A S T E R L Y P R

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T I Y M A K K P V Q L P G Y Y Y V D T K L D I T S

cacaacgaggactacaccatcgtggaacagtacgagcgtccgagggcgccaccacctgttctctgtacggcatg

H N E D Y T I V E Q Y E R S E G R H H L F L Y G M

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K K K L R G F I A E K R C A P L M L R L A F H S A

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D F Y Q L A G V V A V E V T G G P K V P F H P G R

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F G K A M G L T D Q D I V A L S G G H T I G A A H

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ctcgttgacaaatatgcagcggacgaagatgccttcttctgctgattacgctgaggtcaccaaaagctttccgag

L V D K Y A A D E D A F F A D Y A E A H Q K L S E

cttgggtttgctgatgcgcggcgctcatcatcaccaccatttaactcgagtctaga

L G F A D A A A A H H H H H *

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Chapter 3b

Detergent-free labeling of cytoplasmic targets with FLIPPER-bodies for correlated microscopy

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Abstract

Visualization of proteins is essential in cell biology in order to gain insight in protein function. Microscopy is used to locate the proteins in their natural environment using genetically-encoded or affinity-based probes. Genetically-encoded probes modify the target protein, while affinity-based immunolabeling allow the detection of endogenous protein. Immunolabeling of cells however, requires detergents to permeabilize the plasma membrane for intracellular label reagents. These detergents partially dissolve cellular membranes and thereby damage the ultrastructure. Achieving endogenous intracellular labeling with a well-preserved ultrastructure was recently succeeded by a method called NATIVE (nanobody-assisted tissue immunostaining for volumetric electron microscopy). NATIVE uses small fluorescent antigen-binding nanobodies, that can penetrate fixed cells without permeabilization agents. The correlative images between light and electron microscopy can be made based on the fluorescent signal only. A marker visible in electron microscopy (EM) is missing to improve the protein identification at high resolution. The addition of an EM marker was examined by using FLIPPER-bodies in the NATIVE labeling method. FLIPPER-body is a nanobody-based probe fused with a fluorescent protein and peroxidase for correlated microscopy. In a proof-of-concept study anti-GFP FLIPPER-bodies were used to label intracellular GFP fusion proteins in a cell monolayer. Colocalization between target (GFP) and FLIPPER-body (mCherry) showed that indeed, when using a mild primary paraformaldehyde-based fixation, FLIPPER-bodies can penetrate the cytoplasm for intracellular targeting. Although the ultrastructure of these cells is better preserved compared to the ultrastructure in detergent-permeabilized control cells, they seem to suffer from the mild fixation. Thus, while the first proof-of-concept is promising, the right balance between fixative labeling time and probe concentration needs to be further explored. Combining NATIVE and nanobodies visible in both microscopic modalities will result in the best protein identification in correlated microscopy.

Keywords: FLIPPER-body, Detergent-free, Probe, NATIVE, Correlated microscopy

Introduction

Determination of a proteins' localization within cells and tissue, contributes to the knowledge of the proteins' function. Light microscopy (LM) and electron microscopy (EM) can be correlated for multicolor and dynamic protein localization within a high resolution and ultrastructural context in correlated LM and EM (CLEM)¹. In both modalities, protein localization can be achieved via genetically-encoded or affinity-based probes. In genetically-encoded targeting, endogenous proteins are modified². This is in contrast to affinity-based probes, where endogenous proteins can be targeted. Therefore, affinity-based probes are preferred, although the required permeabilization for intracellular targets dissolves cellular membranes³. So, a balance between intracellular labeling and preserving the ultrastructure is important.

Affinity-based immunolabeling is conventionally performed with immunoglobulin type G (IgG) antibodies, which are large, ~150 kDa, multi-chain proteins⁴. Creating a smaller antigen-binding protein would be beneficial in increasing penetration depth and imaging resolution. Therefore, nanobodies were implemented in the development of a new CLEM probe called FLIPPER-body⁵. Nanobodies are the smallest antigen binding proteins derived from heavy-chain only antibodies from Camelidae species^{4, 6, 7} and were genetically fused to FLIPPER (fluorescent indicator and peroxidase for precipitation with EM resolution)⁸. FLIPPER is the combination of a fluorescent protein (FP) and peroxidase to provide fluorescent-based multi target imaging and protein detection at high resolution. The FLIPPER-bodies can be generated in mammalian producer cells and used in an affinity-based immunolabeling approach. The labeling with FLIPPER-bodies indeed showed an improved penetration depth compared to conventional IgGs. However, the ultrastructure is compromised due to using detergent (0.1% triton X-100) for cell permeabilization.

Detergent-free labeling was recently achieved with fluorescent nanobodies in a method called NATIVE (nanobody-assisted tissue immunostaining for volumetric EM)⁹. In this method, 120 μ m vibratome sections of mouse brain were fixed with paraformaldehyde (PFA), followed by a 24-hour labeling with nanobodies conjugated to a chemical dye. After a secondary fixation with glutaraldehyde (GA), it was demonstrated that the fluorescent nanobodies penetrated towards their intercellular target, including cells located deeper inside the tissue. In addition, the ultrastructure was well preserved and organelles were clearly visible. The CLEM data analysis was performed based on the fluorescent probe only. Improved correlation and localization of the protein of interest at EM resolution can be achieved using a probe visible in both modalities because of resolution differences between the microscopes. In this study, the suitability of FLIPPER-bodies as CLEM probes in the NATIVE method is investigated. This would allow large volume imaging directed by the fluorescent signal and high-resolution EM imaging of the labeled protein of interest without damaging the cells/organelles.

Results

Ultrastructural preservation is important in order to get the accurate protein localization within the context of underlying organelles. Therefore, with the NATIVE method, detergents are omitted⁹. In this method, the tissue is fixed with a primary 4% PFA fixation, followed by the

Figure 1 | FLIPPER-bodies can label intracellular GFP without the use of detergents. HEK293T cells expressing EpCAM-GFP were stained with anti-GFP FLIPPER-bodies for 24 hours. By increasing the GA concentration in the primary fixative, the autofluorescence level increased (seen in the red channel). This makes it difficult to distinguish between mCherry signal (FLIPPER-body) and the autofluorescence. Note that in the 4% PFA one GFP positive cell is not stained with the FLIPPER-body. Bars: 20 μ m.

nanobody labeling. Subsequently, the tissue is fixed using a secondary fixative with 2% PFA and 2.5% GA. As a first proof-of-concept experiment, the cell surface protein EpCAM¹⁰ tagged with GFP (EpCAM-GFP) was expressed by HEK293T cells and subsequently fixed with 4% PFA and labeled for 24 hours with anti-GFP FLIPPER-bodies. With light microscopic analysis, a colocalization was seen between GFP target and FLIPPER-body probe in fluorescence (**Figure 1**; 4% PFA). In addition, staining with diaminobenzidine (DAB) precipitation showed the HRP activity in the cells (**Figure 2a**). Note that not all GFP positive cells are labeled with the FLIPPER-body. As additional control, living cells (**Figure 2b**) and fixed wildtype HEK293T cells (**Figure 2c**) were labeled using the FLIPPER-body, but no cells showed a positive labeling. Hence, fixation prior to the staining is necessary for cell permeability.

Addition of GA to the fixative contributes to the ultrastructural preservation in EM³. Therefore, it was examined what the effect of adding GA to the primary fixative was on the labeling efficiency with the anti-GFP FLIPPER-body. By increasing the GA concentration of the fixative, the autofluorescence caused by GA also increased. Especially when using a standard EM fixative (**Figure 1**; 2% PFA with 2% GA), the fluorescence in the red channel is too high to discriminate between the FLIPPER-body and autofluorescence. When decreasing the GA concentration to 0.5%, the staining exceeds the background labeling. However, the staining seems to be more cytoplasmic compared to the cells when GA is omitted, which is caused by

Figure 2 | FLIPPER-bodies do not label intracellular targets in living cells. DIC images taken after DAB staining. **(A)** Fixed (4% PFA) HEK293T cells expressing EpCAM-GFP that were labeled with anti-GFP FLIPPER-bodies for 24 hours. The brown colored cells represent the positive labeled cells. **(B)** EpCAM-GFP expressing HEK293T cells were cultured for 24 hours in conditioned medium containing the FLIPPER-bodies (live-cell). There are no DAB positive cells present. **(C)** Fixed (4% PFA) wildtype HEK293T cells, no GFP expression, are labeled for 24 hours with the anti-GFP FLIPPER-body. No DAB precipitation is visible.

low signal-to-noise ratios. A balance was found in the signal-to-noise and GA concentration at 0.02% GA and therefore this concentration GA is used in the next experiments.

FLIPPER-body visualization of GFP in non-permeabilized cells

FLIPPER-bodies or fluorescent nanobodies are over half the size of IgGs, but less generally applicable because there are limited nanobody targets (Chapter 2, this thesis). Therefore, it was explored whether IgGs can penetrate the NATIVE treated cells. Lysosomal-associated membrane protein 1 (LAMP1)-GFP was used to decorate the outside of the lysosomes with GFP. Cells were either untreated or treated with 0.1% triton X-100 before applying the immunostaining with anti-GFP IgGs (**Figure 3a**) or anti-GFP FLIPPER-bodies (**Figure 3b**). The IgG labeling, 6 hours for each IgG, showed that the IgGs could not penetrate the cells in absence of a permeabilization reagent, thus only GFP was seen (**Figure 3c**). In permeabilized cells the IgGs did enter the cells and colocalize with the lysosomes. Next, FLIPPER-bodies were compared to the IgG immunolabeling using a similar incubation time (6hr). The FLIPPER-bodies stained the intracellular localized GFP in the cells both with and without triton X-100 detergent (**Figure 3d**). Both conditions showed colocalization between LAMP1-GFP and FLIPPER-body. Following LM, the FLIPPER-body stained cells are prepared for EM, including DAB conversion. The ultrastructure in detergent-free cells is well preserved, and the black electron-dense signal generated by DAB, is present at the cytoplasmic side of the lysosomes (**Figure 3e**). However, when using detergent, the ultrastructure was more affected and thereby it was hindering the analysis to determine the organelles to which the DAB signal was associated (**Figure 3e**). This demonstrates that without using detergents small FLIPPER-bodies can penetrate the cells, while large IgGs could not.

Next, anti-GFP FLIPPER-bodies were applied to label actin-GFP, in order to show the probe diversity and to target a non-membrane bound protein. Again, FLIPPER-body labeling was detected in GFP positive cells. However, the actin filaments were less sharp and visible in the FLIPPER-body channel, due to lower signal-to-noise ratios (**Figure 4a**). When proceeding for EM, the actin cytoskeleton of actin-GFP transfected cell was electron-dense (**Figure 4b**), showing the positive FLIPPER-body labeling in a well-preserved ultrastructure. The actin

Figure 3 | FLIPPER-body labeling without compromising the ultrastructure. LAMP1-GFP expressing U2OS cells, labeled with anti-GFP FLIPPER-body or anti-GFP IgG-AF633. **a** Traditional, two-step, indirect IgG labeling conjugated with Alexa Fluor, represented as red star. Antigen is represented as a black triangle. **b** Direct labeling with FLIPPER-body, with an additional secondary step to add electron-dense DAB precipitates, represented here as black circle. **c** Indirect anti-GFP IgG labeling, as represented in **a**. IgGs could not permeabilize the plasma membrane in non-permeabilized cells (-triton). In permeabilized cells, IgG and LAMP1-GFP signals did colocalize (+triton). **d** Direct anti-GFP FLIPPER-bodies targeting lysosomal localized GFP in a direct labeling, as represented in **b**. Non-permeabilized cells (-triton) and permeabilized cells (+triton) resulted both in colocalization between lysosomes and FLIPPER-body. **e** Addition of DAB after FLIPPER-body labeling resulted in black stained lysosomes. Note the well-preserved ultrastructure in non-permeabilized cells (-triton) and dissolved membranes using permeabilizing reagent first (+triton). Bars: LM, 10 μ m; EM, 0.5 μ m. Full EM datasets of -triton and +triton are available at www.nanotomography.org/OA/deBeer2020Thesis/.

Figure 4 | FLIPPER-body staining of actin-GFP in non-permeabilized cells. CLEM of actin-GFP expressing U2OS cells, labeled with FLIPPER-bodies on non-permeabilized cells. a Actin-GFP showed actin fibers present in the cells. FLIPPER-bodies stain the same actin fibers, but less clearly. b Correlated microscopy of cell in a. This cell shows the actin filaments stained in black. The same pattern is seen with LM. In the zoomed in area, details in ultrastructure and filament organization are seen. c Neighboring cell of b without the actin-GFP expression. Not all the cells where GFP positive, and thus this internal control can be used. Here, the actin cytoskeleton is not stained black and only large actin bundles are visible. Note that the entire cell cytoplasm in b is darker compared to the cell in c. Bars: LM and EM overview, 10 μ m; EM zoom, 1 μ m. Full EM data set available at www.nanotomography.org/OA/deBeer2020Thesis/.

cytoskeleton in actin-GFP negative cell (neighboring cell of the actin-GFP positive cell) was visible, but unstained (**Figure 4c**). In addition, only the big actin filaments were visible in the unstained cell. Overall, it is shown that the NATIVE staining method is can successfully be applied using the FLIPPER-bodies, with less damage to the ultrastructure in affinity-based labeling by omitting triton detergents.

No nuclear penetration without permeabilization

Nuclear antibody staining requires more harsh permeabilization or longer incubation times compared to cytoplasmic targets^{3, 5}. Therefore nuclear histone-2B (H2B)-GFP is used to analyze whether FLIPPER-bodies can penetrate the nuclear envelope without the use of detergents. In the absence of permeabilization reagent, FLIPPER-bodies could not access the nucleus to target GFP (**Figure 5**), while in permeabilized cells the FLIPPER-body could

Figure 5 | Nuclear labeling with FLIPPER-bodies require permeabilization. HEK293T cells express nuclear H2B-GFP and are labeled with FLIPPER-bodies. Non-triton permeabilized and triton permeabilized cells were both labeled with FLIPPER-bodies. Only after treatment with detergents, the FLIPPER-body could penetrate the nucleus. Bars: 20 μm .

penetrate the nucleus. This suggests that the nuclear envelope and/or the nuclear matrix does not become as permeable as the plasma membrane and cytoplasm after fixation only.

Immunolabeling with FLIPPER-bodies independent of transfection reagent

The influence of transfection reagent polyethylenimine (PEI) on the NATIVE method was examined because transfection reagents might influence on the cell permeability¹¹. HT29 cells stably expressing EpCAM-GFP were used, where GFP is fused to the C-terminus of EpCAM thus facing the cytoplasm¹². After PFA fixation, the FLIPPER-bodies could penetrate the plasma membrane to label the intracellular localized GFP (**Figure 6**). The EM images also show a positive black staining at the plasma membrane, but because there was no GA used in the primary fixation, the ultrastructure was negatively affected. However, the data implies that using transfection reagents like PEI are not necessary to label according to the NATIVE method.

Discussion and future directions

Labeling proteins for CLEM is often a compromise between good ultrastructural preservation (genetic probes) or labeling at endogenous level using a detergent-based immunolabeling (affinity-based). Recently, Fang and colleagues⁹ presented a new method, NATIVE, in which endogenous proteins were labeled with small fluorescently-tagged nanobody probes without damaging the ultrastructure by detergents. In this proof-of-concept study, FLIPPER-bodies were applied using the NATIVE protocol to have both a fluorescent and electron dense marker present.

FLIPPER-bodies were successfully applied using the NATIVE method. In this NATIVE method, cells are primarily fixed with PFA after which they are labeled by 24-hours, followed by a secondary fixation step with 2% PFA and 2.5% GA. In the current study, the labeling time was decreased to 6 hours since only monolayers of cells were targeted. The decreased labeling time leads to earlier EM embedding, and thus better a preservation of the ultrastructure. For a good ultrastructure in EM (intact membranes, no extraction artifacts, no holes), GA is typically added in the concentration range between 0.2% and 2%. With these concentrations

Figure 6 | FLIPPER-body staining in stable expressing HT29. Stably expressing EpCAM-GFP expressing HT29 cells were fixed with 4% PFA and labeled for 24hr with FLIPPER-bodies, which showed colocalization at the plasma membrane between GFP and mCherry. The plasma membrane of all cells has the black precipitate after DAB conversion. From the zoomed in area, it can be appreciated that the black staining is intracellular. Bars: LM, 10 μ m; EM overview, 5 μ m; EM zoom, 0.5 μ m. Full EM data sets available at www.nanotomography.org/OA/deBeer2020Thesis/.

however, FLIPPER-body signals were hard to detect because of GA autofluorescence interferes in the mCherry channel. Here, a concentration of 0.02% GA was added to the primary fixative to have the right balance between labeling efficiency and ultrastructure preservation.

Despite the addition of GA to the primary fixative, it was noticed that not all GFP positive cells were labeled with the FLIPPER-body in cells fixed with PFA. This raises the question in the original NATIVE method all cells and proteins were labeled or whether this decreased labelling is caused size differences between the fluorescent nanobody and FLIPPER-body. In the original paper presenting the NATIVE method, a nanobody fused with a small chemical dye (~16kDa) was used to label endogenous proteins. Hence, negative stained cells might have been missed. Therefore, it is recommended to further examine the effect by decreasing the FLIPPER-body probe size, by omitting mCherry or HRP.

The FLIPPER-body labeling did result in background staining, seen as dark stained cytoplasm, especially noticeable when targeting actin-GFP (Figure 4). Actin is present in the cells as free monomers, before being incorporated in larger actin fibers¹³. Therefore, free actin-GFP is present in the cytoplasm and can already be detected with the FLIPPER-bodies. This would explain why the actin fibers are less visible with the FLIPPER-bodies. However, in EM, the actin fibers are well stained, which is contrary to the LM result with FLIPPER-bodies. When comparing the cytoplasm of a positive labeled cell and a negative labelled cell in EM, it is noted that the entire cytoplasm in the positive cell is a bit darker. For that reason, tagging proteins with genetically-encoded probes might be better for a high signal-to-noise ratio with increased labeling sensitivity and specificity, compared to affinity-based labeling. Using gene editing tools will contribute to tag the proteins of interest at endogenous expression

levels with very high efficiency, but these gene editing tools are more laborious than affinity-based probes^{14, 15}.

Affinity-based labeling has the advantage of tagging endogenous proteins without changing the protein size, and thereby potentially its function. However, high background labeling is seen with conventional fluorescence IgG labeling when a too high probe concentration is used. This might hint toward using a too high FLIPPER-body concentration in this study as well. In a $n=1$ experiment (data not shown) based on EM data, the background seemed to be reduced when adding diluted FLIPPER-body reagent. The used conditioned medium though, varies a lot in the FLIPPER-body concentration each time the probe is generated. Therefore, to better control the used concentration, FLIPPER-bodies should be isolated first using the present HIS-tag. Once the FLIPPER-body concentration is determined, a dilution series will help finding the best concentration for a good signal-to-noise.

In order to exclude the effect of transfection reagent PEI on the cell permeability, stable EpCAM-GFP expressing cells were labeled with FLIPPER-bodies after fixation. PEI transfection is used to deliver the cDNA intracellular via endocytosis. Interactions between PEI and lipid membrane might influence the membrane composition and therefore induce cell permeability¹¹. In this study, it is shown with stable GFP expressing cells that NATIVE labeling is independent of PEI transfection, suggesting that PEI itself is not required for the NATIVE labeling with FLIPPER-bodies. In addition, live-cells with intracellular GFP were not labeled with the FLIPPER-bodies, indicating that fixation caused the cell permeability. Other studies already showed an effect of PFA fixation on (i) the cell morphology, showing protein extraction and damaged membranes^{3, 16} and (ii) on a decrease in cell mass¹⁷. Most of the lipids are not immobilized by the PFA fixation and lipids might be dissolved^{18, 19}, resulting in the above mentioned phenomena contributing to cell permeability.

In conclusion, FLIPPER-bodies were used somewhat successful in the NATIVE staining method, in which cells are fixed in PFA with a low GA concentration before being labeled. The ultrastructure is better preserved compared to detergent-based labeling based on membrane preservation. However, the cells still suffer from a long labeling time in combination with low percentages of GA in the primary fixative. This is mainly reflected as empty areas within the cells. On top of these artefacts, the major concern of the labeling is that it does not stain all GFP positive cells. It is therefore concluded that, although the method appears promising, it requires further developments to be generally applicable to labeling proteins. In its current state of development, it performs worse than existing post-embedding affinity-based labeling on cryo- (Tokuyasu method^{20, 21}) or Epon sections¹ with regard of ultrastructure preservation. Since post-embedding labeling (mainly Epon) has a very limited number of positive targets, further development of the current method appears attractive. Furthermore, nanobodies could potentially be beneficial in application in both Tokuyasu and Epon labeling, since they exhibit better cell penetration, and have a higher labeling efficiency (Chapter 2, this thesis). In addition, pre-embedding genetic CLEM probes, like APEX2 with GFP²² or FLIPPER⁸ also have a well-preserved ultrastructure and have more trusted data concerning labeling efficiency of all the cells. Therefore, before the detergent-free method can be successfully applied, further development is required in finding the right fixative, FLIPPER-body concentration, probe size and labeling time.

Acknowledgment

We acknowledge all labs that made their constructs available through Addgene (E. Dell'Angelica and G. Wahl) and our lab members for feedback and discussion; financial support by The Netherlands organization for scientific research (NWO 175-010-2009-023; ZonMW 91111.006; STW Microscopy Valley 12718) and Jan Kornelis de Cock Stichting.

Methods

Cell culture

HEK293T, U2OS and HT29 EpCAM-GFP cells were cultured in DMEM (Gibco, 31966-021), supplemented with 5 % Fetal Bovine Serum (FBS, Greiner bio-one, 758093) and penicillin/streptomycin (PAA, P01011-1692), at 37 °C in the presence of 5 % CO₂. Cells were transfected using 1:4,5 DNA / polyethyleneimine 1 mg/ml (PEI MW 25,000, Polysciences, 23966) for 48 hours. For imaging, cells were seeded on glass bottom Petri dishes (Greiner, 627870) that are, before cell seeding, sputter coated with 2 nm palladium/gold (LeicaSCD050), after which a pattern was made by scratching with a needle to enable CLEM.

Plasmids

The following plasmids were used: pcDNA3.1 anti-GFP FLIPPER-body (mCherry – HRP) [addgene plasmid # 112157]⁵; pcDNA3.1 LAMP1-GFP [adapted from E. Dell'Angelica: addgene plasmid # 34831]²³; pcDNA3.1 Histone 2B (H2B)-GFP [adapted from G. Wahl: addgene plasmid # 11680]²⁴; pcDNA3.1 EpCAM-GFP¹² and pcDNA3.1 Actin-GFP.

Primary fixation

Cells were fixed by adding an equal volume of fixative (4% paraformaldehyde (PFA, Merck, 1.04005.1000) and 0.02% glutaraldehyde (GA, Polysciences, 01909-100) in 0.1 M sodium cacodylate buffer (pH 7.4, Sigma, C0250-500g) to the medium. After 10 minutes, this mixture was replaced with the above fixative solution only, for 45 min and rinsed twice with PBS (pH 7.4). All steps were performed at RT. Detergent-treated control cells were fixed using 4% PFA and 0.2% GA.

FLIPPER-body generation

FLIPPER-bodies were produced by transiently transfected HEK293T cells. After 48 hours, conditioned media was collected and tested on secreted HRP activity by mixing 1:1 with diaminobenzidine (DAB) solution. DAB solution was made by adding 5 mg DAB (Sigma, E7750) in 10 ml PBS and filter the solution through a Whatman 0.2 µm filter, after which a 3 µl of 30% H₂O₂ (Sigma, H1009) was added. A color change to brown was noticed in the presence of FLIPPER-bodies.

Labeling of cells and confocal microscopy

Control cells were treated with 0.1 % Triton X-100 (Sigma, X100) for 8 min and rinsed 2x5 min with PBS. Next, all cells were incubated in 1% bovine serum albumin (BSA, Roche, 10735086001) in PBS for 30 min, after which cells were stained for 6 hours with fresh FLIPPER-bodies (conditioned media) or with primary anti-GFP IgGs (1:200 in 1% BSA, Rockland, 600-401-215). Cells were washed 3x10 min with PBS. Cells stained with the IgG were incubated additionally with a secondary goat anti-rabbit IgG conjugated to Alexa Fluor 633 (AF 633) (1:500 in 1% BSA, Thermofisher, A-21070) and finally rinsed 3x10 min with PBS. All steps were performed at RT.

Fluorescent and DIC images were generated on the same day as labeling, using confocal microscopy (Zeiss LSM 780, Plan-Neofluar 63x/N.A. 1.3 Imm Korr DIC M27 lens) while the

cells were in PBS. After imaging, cells were post-fixed with 2% PFA and 2% GA in 0.1 M sodium cacodylate overnight at 4°C.

DAB polymerization, embedding and electron microscopy

After post fixation samples were rinsed 3x5 min with PBS and stained with DAB for 15 min (as described above). Afterward the cells were rinsed 3x5 min with PBS. Samples were embedded in Epon as described previously²⁵. The glass bottom was dissolved using hydrogen fluoride (Sigma, 01066) for 10 min.

The Pd/Au pattern in the Petri dish was used to find the regions of interest selected in confocal microscopy. Ultrathin sections (80nm) of these regions were obtained and collected on single-hole copper grids (Electron Microscopy Sciences, A1000-Cu). Sections were not contrasted with uranyl and lead. The regions of interest were stabilized in an electron beam using a TEM (FEI, CM100) at 80 keV. Next, large-scale area scans (nanotomography), with 2.5 nm pixel size, were generated using the STEM detector in a Zeiss Supra55 scanning electron microscope at 28 keV^{25, 26}. Images were analyzed and processed in Adobe Photoshop. All datasets are available at www.nanotomography.org/OA/deBeer2020Thesis/.

3b

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Chapter 4

Endocytosis of extracellular vesicles and release of their cargo from endosomes

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ACS Nano (2020) 14(4):4444-4455
doi: 10.1021/acsnano.9b10033

Abstract

Extracellular vesicles (EVs), such as exosomes, can mediate long-distance communication between cells by delivering biomolecular cargo. It is speculated that EVs undergo back-fusion at multivesicular bodies (MVBs) in recipient cells to release their functional cargo. However, direct evidence is lacking. Tracing the cellular uptake of EVs with high resolution as well as acquiring direct evidence for the release of EV cargo is challenging mainly because of technical limitations. Here we developed an analytical methodology, combining state-of-the-art molecular tools and correlative light and electron microscopy (CLEM), to identify the intracellular site for EV cargo release. GFP was loaded inside EVs through the expression of GFP-CD63, a fusion of GFP to the cytosolic tail of CD63, in EV producer cells. In addition, we genetically engineered a cell line which expresses anti-GFP fluobody that specifically recognizes the EV cargo (GFP). Incubation of anti-GFP fluobody-expressing cells with GFP-CD63 EVs resulted in the formation of fluobody punctae, designating cytosolic exposure of GFP. Endosomal damage was not observed in EV acceptor cells. Ultrastructural analysis of the underlying structures at GFP/fluobody double-positive punctae demonstrated that EV cargo release occurs from endosomes/lysosomes. Finally, we show that neutralization of endosomal pH and cholesterol accumulation in endosomes leads to blockage of EV cargo exposure. In conclusion, we report that a fraction of internalized EVs fuse with the limiting membrane of endosomes/lysosomes in an acidification-dependent manner, which results in EV cargo exposure to the cell cytosol.

Keywords: Extracellular vesicles, Endosomes, Nanobody, Endosomal escape, Cargo delivery, Correlative microscopy

Introduction

Secreted factors, including messenger molecules and extracellular vesicles allow long-distance communication between mammalian cells. Extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, carry biomolecules (lipids, proteins, nucleic acids) and impart phenotypic changes in recipient cells. EVs have been reported to play a role in a wide variety of processes within the human body, including immune response, neurodegenerative disease pathogenesis, viral dissemination, and tumor formation and metastasis¹⁻⁸. Next to their role in cell-cell communication, EVs show promise as biological

drug delivery vehicles⁹⁻¹¹. Multiple types of EVs exist, which are firstly categorized on the basis of their biogenesis: (i) Microvesicles and (ii) apoptotic bodies are generated by plasma membrane outward budding, whereas (iii) exosomes are created by endosome membrane invagination, resulting in intraluminal vesicles within multivesicular bodies (MVBs). Subsequent fusion of MVBs with the plasma membrane results in exosome release from cells. EVs can enter cells in the local or distant environment^{1, 12-18}, via fusion and/or endocytosis¹⁹⁻²⁴.

Figure 1 | Experimental set-up to elucidate the intracellular site of EV-cargo release. EVs interacting with recipient cells can release their cargo via: i. direct fusion with the plasma membrane. ii. kiss and run fusion with the endoplasmic reticulum. iii. fusion with the endosome membrane. iv. endosomal rupture. (A) In cells engineered to cytosolically express monomeric azam-green galactin-3 fusion protein (mAG-gal3), mAG-gal3 punctae formation only occurs in case of endosomal rupture (iv). (B) In cells engineered to cytosolically express mCherry-tagged anti-GFP fluobody, mCherry punctae formation only occurs in case of fusion of GFP-CD63 EVs (i.e., membrane-bound GFP inside the EV) with the plasma membrane (i) or endosome membrane (iii).

Different mechanisms for EV cargo release in recipient cells have been proposed, including (i) fusion with the plasma membrane^{19, 20}, (ii) kiss and run fusion with the endoplasmic reticulum²¹, (iii) fusion with the endosome membrane²² and (iv) endosomal rupture (Figure 1)^{22, 25, 26}. Although fusion of EVs with the plasma membrane of recipient cells has been proposed as a mechanism for content release^{19, 20}, endocytosis is the major pathway of EV uptake²¹⁻²⁴. Escape of the EV content from the endosomal confinement is then a requirement for its functionality as it needs to access cytoplasmic targets in the host cell, such as the RNA-induced silencing complex (RISC) machinery for miRNAs. Possible mechanisms for cargo release of EVs from endosomes include endosomal lysis, endosomal permeabilization, and membrane fusion between EV and endosomal membrane²⁷.

The incidence of cytosolic delivery of EV cargo has been largely indirectly inferred

from functional studies in which e.g. EV-mediated delivery of miRNA was shown to result in altered gene expression^{15, 28-32}. However, direct evidence for EV content release into the cytosol of recipient cells is lacking. Here, we employed state-of-the-art molecular tools with correlative light and electron microscopy (CLEM)³³ to detect endosomal lysis and identify the underlying ultrastructure of intracellular sites of EV cargo exposure to the cell cytosol, following EV uptake in HEK293T cells. To this end, fluorescent GFP-CD63 EVs were generated, carrying GFP at the interior EV membrane. Cytosolic expression of anti-GFP fluobody³⁴, an mCherry-tagged anti-GFP single-domain antibody (nanobody)^{35, 36}, in recipient cells was exploited to detect EV cargo exposure to the cytosol (**Figure 1**).

Results and Discussion

Extracellular vesicles are internalized via endocytosis

In order to study the processing of exogenously added EVs in mammalian cells by fluorescence light microscopy (LM), a stable GFP-CD63 HEK293T cell line was generated for the production of fluorescently labeled EVs. In GFP-CD63 HEK293T cells, GFP fluorescence showed cell surface staining and a punctate staining pattern consistent with the cytoplasmic distribution of endosomes (**Figure S1A**), which corresponds with the localization of endogenous CD63³⁷. EVs were isolated by differential centrifugation of the conditioned cell culture medium, with final ultracentrifugation at 100,000g (small EVs). Following isolation, both wildtype (WT) and GFP-CD63 EVs showed cup-shaped vesicular morphology and a diameter of 100-150 nm, by electron microscopic investigation (**Figure S1B**). WT and GFP-CD63 EVs displayed a similar extent of enrichment of EV marker proteins and low levels of the Golgi protein golgin-97, an EV negative marker, in comparison to the respective parent producer cells (**Figure S1C**). Furthermore, size distribution analysis using dynamic light scattering confirmed the similar size of WT and GFP-CD63 EVs and also their surface charge (ζ -potential) was shown to be identical (**Figure S1D - F**). Hence, GFP-CD63 expression did not alter morphology nor size or surface charge of the EVs. Therefore, GFP-CD63 EVs were considered similar to WT EVs, and were further used in the study.

Upon incubation of WT HEK293T cells with GFP-CD63 EVs, a punctate staining pattern was observed throughout the cytosol by LM suggesting the involvement of endocytosis in EV uptake by cells (**Figure 2A**). Indeed, inhibition of endocytosis through the use of the dynamin inhibitor dynasore³⁸, resulted in a decrease in EV uptake (**Figure S2A**). In addition, EV uptake was inhibited at a non-permissive temperature (4 °C) for endocytosis (**Figure S2B**). Taking a CLEM approach allowed for the identification of the ultrastructure of the GFP-positive spots by EM (**Figure 2B, C**), revealing the presence of GFP-CD63 EVs in membranous compartments, i.e., endosomes (**Figure 2C, Figure S3**). To confirm the presence of GFP-CD63 EVs within these endosomal structures, GFP was immunolabeled and detected with a secondary antibody conjugated to QD655. Indeed, the endosomes that were identified by EM (**Figure 2C**) and appeared positive for GFP by LM examination (**Figure 2B**), were also found positive for GFP after immunolabeling (**Figure 2D**). Taken together, the findings demonstrate that GFP-CD63 EVs are taken up by HEK293T cells via endocytosis. Of note, not all compartments that were positive for GFP in the CLEM image stained positive for GFP upon immunolabeling. This can be explained by the low efficiency of EM immunolabeling in general³⁹.

Figure 2 | Exogenously added EVs localize in membrane-bound compartments in HEK293T acceptor cells. (A) HEK293T cells incubated for 12 hours with GFP-CD63 EVs show a punctate staining pattern in the cytosol. Bars: 10 μm . **(B)** Correlative light (green) and EM (greyscale) microscopy for ultrastructural analysis of the internalized EVs (GFP punctae) of the boxed area in A. Bar: 5 μm . **(C)** Underlying ultrastructures of areas 1-3 in B reveal vesicular structures. Additional snap shots available in Supplementary Fig. 3B. Bar: 0.2 μm . **(D)** Structures given in C are labeled with QDs (indicated by arrowhead) following anti-GFP immunolabeling, confirming the presence of GFP-CD63 EVs within the vesicular structures. Bar: 0.2 μm . All EM datasets at full resolution are available via www.nanotomym.org.

Extracellular vesicles do not induce endosomal permeabilization

Following endocytosis of EVs by HEK293T cells, a prerequisite for delivery of their cargo to the cell cytosol is their escape from endosomal confinement. To address whether EVs permeabilize the endosomal membrane to escape from endosomes, a galectin-3 based assay was used. For this purpose, HEK293T cells were transduced to express monomeric Azami green-tagged galectin-3 (mAG-gal3) in their cytosol. mAG-gal3 identifies damaged endosomes by binding to β -galactosides that are present at the luminal leaflet of endosomes, which results in the formation of mAG-gal3 punctae (**Figure 3A**)⁴⁰. To address whether mAG-gal3 punctae (green) are formed upon incubation of HEK293T cells with EVs, mAG-gal3 HEK293T cells were incubated with red fluorescent CD63-RFP EVs (**Figure S4**).

A diffuse cytosolic green fluorescence without punctae was observed in mAG-gal3 expressing HEK293T cells in the presence of CD63-RFP EVs for 12 hour, which was similar to

Figure 3 | EVs do not induce endosomal permeabilization. (A) Cartoon illustrating the galectin-3-based assay to detect endosomal permeabilization. HEK293T cells are engineered to cytosolically express monomeric azami-green galectin-3 fusion protein (mAG-gal3). Upon endosomal permeabilization, galactoside residues at the inner leaflet of the endosome are accessible to mAG-gal3, resulting in mAG-gal3 accumulation in the endosome and puncta formation. (B) Fluorescence images of mAG-gal3 expressing HEK293T cells untreated (control), treated with CD63-RFP EVs (EV) and transfected with Lipofectamine (Lipo) (t=12hours). Red, EVs; green, mAG-gal3; blue, nucleus. Bars: 10 μ m. (C) Quantification of CD63-RFP EV uptake (red line) and mAG-gal3 punctae formation (green line) in cells over time (error bars represent SD, n=3, $\geq$ 36 cells analyzed per time point). (D) Quantification of mAG-gal3 punctae upon 12 hours treatment of mAG-Gal3 HEK293T cells with EVs and Lipofectamine-based lipoplexes. mAG-gal3 punctae only appear in cells with lipoplex treatment (n=3; $\geq$ 45 cells analyzed per condition; **P < 0.01, ***P < 0.001, ns; not significant, ANOVA Tukey's post hoc test).

in untreated cells (Figure 3B). As a positive control, cells were incubated with Lipofectamine-based lipoplexes, which are known to disrupt endosomes⁴⁰. Indeed, multiple mAG-gal3 punctae were formed in cells treated with lipoplexes (Figure 3B, quantified in D). To exclude the possibility that mAG-gal3 punctae were present at earlier time points but disappeared during the prolonged incubation time, mAG-gal3 cells were incubated with RFP-tagged EVs for 2, 4, 8 and 12 hours (Figure S5), after which EV uptake and mAG-gal3 punctae were quantified. Significant uptake of EVs by mAG-gal3 HEK293T cells was seen after 2 hours of incubation. A plateau in EV uptake was reached after 4 hours incubation, showing 66 ± 9 RFP-positive spots per cell, indicating that EVs were efficiently internalized by mAG-gal3 cells (Figure 3C, red line). However, a very limited number of galectin punctae was observed at all of the investigated time points (Figure 3C, green line), which was similar to the number of punctae in untreated (control) cells (Figure 3D). Moreover, no colocalization was observed between CD63-RFP EVs and mAG-gal3 punctae at any of the investigated time points. Live cell imaging of mAG-gal3 HEK293T cells incubated with CD63-RFP EVs for 4 hours confirmed the absence of mAG-gal3 punctae formation, indicating that such events did not remain undetected because of a transient nature of the mAG-gal3 punctae, nor that punctae were absent due to fixation-induced artifacts (Movie 1 and still images in Figure S7)⁴¹. A five-times increase in the EV concentration with the mAG-gal3 cells neither resulted in mAG-gal3 punctae formation (Figure S6). Collectively, the data show that internalized EVs do not permeabilize endosomes in HEK293T cells.

Extracellular vesicles release their cargo from endosomes/lysosomes

Next, we considered the involvement of non-disruptive EV-endosome membrane fusion in the unloading of EV cargo. To visualize EV cargo release into the cell cytosol we used a fluorescent membrane-bound cargo, because a soluble cargo would rapidly dilute in the cytosol, resulting in a fast loss of signal, precluding further investigation by means of CLEM. Specifically, GFP-CD63 EVs with GFP present at the N-terminal end of CD63, i.e., inside the EVs, were used (**Figure S4A**). First, the presence of GFP at the inside of EVs was verified. To this end, GFP-CD63 EVs were immuno-stained with anti-GFP antibody and secondary antibody conjugated to gold without prior permeabilization. WT EVs were used as a negative control, and EVs with GFP fused to the extracellular loop of CD63 (outGFP-CD63 EVs) were used as a positive control. OutGFP-CD63 EVs showed a positive signal in immunostaining, whereas WT and GFP-CD63 EVs showed no signal (**Figure S4D**). These data confirm that GFP when fused at the N-terminal end of CD63 is not accessible to the anti-GFP antibody and thus present at the inside of the EVs.

Then, HEK293T cells were engineered to express an anti-GFP nanobody fused to mCherry (anti-GFP fluobody) in the cytosol. Upon fusion of GFP-CD63 EVs with the endosomal membrane, GFP that is present inside the EVs, would become exposed to the cytoplasm. Consequently, the cytosolic anti-GFP fluobody would identify such a fusion event (**Figure 4A**). Upon the incubation of fluobody-expressing HEK293T cells with GFP-CD63 EVs, mCherry punctae were formed, designating the cytosolic exposure of EV cargo (**Figure 4B**). Fluobody punctae were present in nearly all cells ($94 \% \pm 4.4 \%$; **Figure S8**) after 12 hours. In addition, maximum colocalization (yellow) between EVs (green) and fluobody punctae (red) was seen after 12 hours of incubation, when $24 \% \pm 1.2 \%$ of GFP spots colocalized with mCherry punctae (**Figure 4B, C**). Importantly, mCherry punctae always colocalized with GFP spots, validating the specificity of the anti-GFP fluobody. Moreover, incubation of fluobody-expressing cells with WT EVs revealed the absence of mCherry punctae, confirming the specificity of mCherry puncta formation towards GFP-CD63 EVs (**Figure S9**). Of note, the maximum EV/fluobody colocalization at $t=12$ hours doesn't mean that maximum cargo exposure occurs at this time point. It more likely reflects persistence over time of the structures from where release has taken place. Next, to identify the underlying ultrastructure at the sites of colocalization between GFP-CD63 EVs and fluobody punctae, CLEM was performed. To maximize the chance of detecting sites of EV-fluobody colocalization, mCherry-fluobody HEK293T cells that were incubated with GFP-CD63 EVs for 12 hours were investigated. Ultrastructural analysis revealed the presence of late endosomes/MVBs and lysosomes at the intracellular sites of colocalization between EVs and fluobody punctae (**Figure 4D, E**). This implies that EV cargo is released from late endosomes and lysosomes and/or that EV cargo is released from (early) endosomes that subsequently undergo maturation. To confirm the identity of the endosomal structures as revealed by CLEM investigation, fluobody-expressing cells were incubated with GFP-CD63 EVs for 12 hours and immunostained for LAMP1, a marker for both late endosomes/MVBs and lysosomes. The extent of colocalization between EV/fluobody spots and LAMP1 was $43 \pm 13 \%$ (**Figure 4F**). Altogether, the data demonstrate that a fraction of internalized EVs undergoes fusion with endosomes and/or lysosomes, resulting in EV cargo exposure to the cell cytosol.

Figure 4 | EV cargo release occurs from endosomes. (A) Cartoon illustrating the experimental design to identify EV cargo exposure to the cell cytosol. When GFP-CD63 EVs in endosomes undergo fusion with the endosome membrane in anti-GFP fluobody (mCherry) HEK293T cells, the anti-GFP fluobody can access and recognize the GFP at the EV interior, resulting in formation of mCherry punctae. (B) Fluorescence images of anti-GFP fluobody (mCherry) HEK293T cells incubated with GFP-CD63 EVs for 4, 8 and 12 hours. Yellow punctae represent colocalization. Green, EV; red, fluobody. bars: 10 μ m. (C) EV uptake (green line) and colocalization with fluobody punctae (yellow line). Colocalization of GFP and mCherry indicate EV cargo exposure to the cell cytosol (error bars represent SD, n=3, ≥ 36 cells analyzed per time point). (D) Correlative light (red + green) and EM (greyscale) microscopy of anti-GFP fluobody (mCherry) cells incubated with GFP-CD63 EVs for 12 hours. Numbers 1-4 indicate areas of red and green (yellow) colocalization. Bars: 2 μ m. (E) The underlying ultrastructure of intracellular sites of EV cargo exposure to the cell cytosol (areas 1-4 in D). Membrane-bound structures containing numerous ILVs (1, 3 and 4) represent MVBs, while structures with electron-dense interior (2) represent lysosomes. Bars: 0.2 μ m. Complete dataset at maximum resolution is available at www.nanotomy.org. (F) Immunostaining for the late endosome/lysosome marker LAMP1 in anti-GFP fluobody cells incubated for 12 hours with GFP-CD63 EVs. Green, EV; red, fluobody; 633, antibody staining color-coded blue. Bars: 10 μ m.

Figure 5 | EV cargo release is blocked by treatment of acceptor cells with the V-ATPase inhibitor Bafilomycin A1 or U18666A, an inhibitor of cholesterol export from late endosomes/lysosomes. (A) Anti-GFP fluobody (mCherry) expressing cells were incubated for 12 hours with GFP-CD63 EVs in the absence and presence of BafA1 or U18666A. Note that colocalization (yellow) between EVs (green) and fluobody punctae (red) is largely absent in BafA1 and U18666A treated cells. Green, EV; red, fluobody; blue, nucleus. Bars: 10 μ m. **(B)** Quantification of colocalization of GFP-CD63 EVs and fluobody punctae after 12 hours incubation in the absence and presence of BafA1 or U18666A. Number of GFP/mCherry double-positive spots in control cells is set at 100 % (error bars indicate SD, n=3, $\geq$ 24 cells analyzed per condition; *P < 0.05, **P < 0.001, two-tailed Student's t-test).

Neutral pH and elevated cholesterol in endosomes block EV content release from endosomes

Viral fusion with endosomes has been shown to be pH- and cholesterol-dependent⁴². To address whether these factors also control EV content release from endosomes, two metabolic inhibitors, Bafilomycin A1 (BafA1) and U18666A, were used. BafA1 is a V-ATPase inhibitor that prevents the acidification of endosomes during endosomal maturation⁴³. In the presence of BafA1, fluobody punctae formation was almost completely abolished in anti-GFP fluobody (mCherry)-expressing cells that were treated with EVs (**Figure 5A**). The overlap between GFP-CD63 EVs and fluobody punctae decreased nine-to-tenfold as compared to untreated (control) cells (**Figure 5B**). Importantly, treatment with BafA1 did not affect cell viability and EV internalization (**Figure S10A, B**), while it effectively prevented endosomal acidification (**Figure S10C**). Treatment of cells with U18666A, an inhibitor of lysosomal cholesterol export, has been shown to trigger cholesterol accumulation in late endosomes and lysosomes^{44,45}. Incubation of anti-GFP fluobody HEK293T cells with EVs in the presence of U18666A resulted in a reduction in fluobody punctae compared to incubation in the absence of U18666A (**Figure 5A**), while the colocalization between fluobody punctae and GFP-CD63 EVs showed a five-to-six-fold decrease (**Figure 5B**). Treatment of HEK293T cells with U18666A resulted in enlarged LAMP1-positive compartments, and cholesterol accumulation in endosomes (**Figure S10D, E**, respectively). Collectively, the data indicate that EV-endosome membrane fusion is inhibited by neutralization of endosomal pH and cholesterol accumulation in endosomes, which prevents EV content release from endosomes into the cytosol.

Conclusions

Extracellular vesicles (EVs), including exosomes, enable (long-distance) cell-cell communication. Their potential use for disease diagnosis and drug delivery is extensively investigated. However, the mechanism of EV-mediated cargo transfer between cells remains largely obscure. Different sites for EV cargo release in acceptor cells have been proposed, including (i) the plasma membrane¹⁹, (ii) the endosome^{22, 25}, and (iii) the endoplasmic reticulum²¹. In this study, an analytical methodology was developed to visualize EV uptake and cargo release in acceptor cells, in order to identify the intracellular site for EV cargo release (**Figure 1**). Using correlative light and electron microscopy (CLEM), a combination of fluorescence microscopy and EM, we reveal the ultrastructural context of cellular structures containing fluorescently labeled EVs (**Figure 2**). EV localization in endosomes and lysosomes confirmed their uptake by endocytosis in line with hitherto reported studies²¹⁻²⁴.

Escape from endosomal confinement, is necessary for EVs to expose their cargo to the cytosol. Studying the fate of EV cargoes, has been challenging owing to the low quantities of encapsulated cargo molecules^{18, 46}. Despite the development of tools to enhance EV loading efficiency, direct proof for cargo release is lacking^{23, 24}. Recently, EV cargo release from endosomes was examined by the use of GFP-carrying EVs labeled with a quenching concentration of R18²². Dequenching of the R18 probe at the level of late endosomes/MVBs revealed membrane interaction between exosomes and late endosomes/MVBs, indicative for cargo release at late endosomes/MVBs. However, dequenching of R18 shows dilution of exosomal lipids and cannot distinguish endosomal rupture from membrane fusion. Moreover, R18 dilution does not demonstrate cargo exposure to the cytosol, and release of GFP content from endosomes into the cytosol was not detected in the study²², possibly because release of soluble GFP cargo remains undetected because of its rapid dilution in the cytosol. In the present study, to increase the chance of detecting EV cargo exposure to the cytosol of acceptor cells, membrane-bound EV cargo (i.e. GFP-CD63) was used. Formation of mCherry punctae in mCherry-tagged anti-GFP nanobody-expressing HEK293T cells upon their exposure to GFP-CD63 EVs revealed GFP exposure, i.e., EV cargo delivery, to the cytosol (**Figure 4B, C**). Examination of the underlying ultrastructure at GFP/mCherry double-positive punctae, revealed late endosomes/MVBs and lysosomes (**Figure 4D, E**). This finding was corroborated by a positive immunostaining for the late endosome/MVB and lysosome marker LAMP1 at the double-positive punctae (**Figure 4F**). Of note, mCherry punctae were never detected at the plasma membrane, suggesting that EV fusion with the plasma membrane (**Figure 1B**; i) does not occur. However, taking into consideration the possibility of rapid diffusion of lipids and proteins at the plasma membrane, a failure to capture fluobody punctae at the plasma membrane cannot be ruled out. Therefore, with the presented data, we do not rule out the possibility of direct fusion of EVs with the plasma membrane.

Endosomal permeabilization as the mechanism of EV cargo release was excluded, because of the absence of mAG punctae in EV-treated HEK293T acceptor cells expressing cytosolic mAG-gal3 (**Figure 3**), specifying EV-endosome membrane fusion as a mechanism behind EV cargo delivery to acceptor cells. Of note, only a fraction of the EVs that were internalized by cells exposed their cargo to the cytoplasm, varying from 10 % after 2 hours to 24.5 % after 12 hours of incubation. This shows that functional delivery of EV cargo is limited. The design

of EVs with a higher propensity to fuse with (endosomal) membranes will facilitate the advancement of improved EV-based therapeutics. Such approaches may include insertion of cell penetrating peptides (CPPs), (viral) fusogenic peptides/proteins, and cationic lipids, and are currently under investigation⁴⁷⁻⁵¹. Moreover, surface functionalization of EVs to alter their tissue tropism will enhance cell specificity and prevent off-target effects. Combining the advantages of EVs, i.e., a biological delivery system, and synthetic delivery systems provides opportunities to improve target specificity, safety, and efficiency in drug delivery⁵¹⁻⁵⁵.

EV entry by endocytosis and subsequent cargo release via membrane fusion suggests that EVs exploit mechanisms akin to certain viruses^{19, 56}. These viruses exploit a low pH-induced change in the tertiary structure of viral envelope proteins to induce fusion with acidified endosomal compartments⁴². A reduction in EV cargo delivery to cytosol after inhibition of endosomal acidification by Bafilomycin A1, showed that EVs respond to low pH for undergoing membrane fusion with endosomal membranes (**Figure 5**). Further investigation is required to see if low pH can alter membrane characteristics of the internalized EVs rendering them susceptible for membrane fusion. Importantly, at different stages of maturation endosomes differ not only in their luminal pH but also in lipid and protein composition^{57, 58}. Anionic lipids in late endosomes have been shown to act as co-factors for fusion of viruses with the endosomal membrane⁵⁹. Likewise, the endosomal escape of genetic cargo mediated by synthetic gene delivery vectors was shown to occur from maturing endosomes⁶⁰, initiating discussion on the involvement of a specific class of anionic lipids in mediating endosomal escape^{61, 62}. U18666A treatment is well known to trigger accumulation of cholesterol and the anionic lipid LBPA in maturing endosomes^{63, 64}. In the present study, a reduction in the number of fluobody punctae upon treatment of anti-GFP fluobody expressing HEK293T cells with GFP-CD63 EVs in the presence of U18666A (**Figure 5**) revealed that EV cargo release was significantly inhibited in the presence of the cholesterol transport inhibitor U18666A, even though endosomal compartments in U18666A-treated cells were acidic in nature, as indicated by the presence of Lysotracker (**Figure S10**). This may suggest that the lipid composition of the endosomal membrane plays a role in EV-endosome membrane fusion.

Taken together, using a sophisticated approach that combines CLEM, cytosolic expression of mAG-gal3 that detects endosomal damage, and cytosolic expression of a fluorescently tagged probe that recognizes EV cargo, we provide experimental proof for the exposure of EV cargo to the cytosol via fusion of EVs with endosomes/lysosomes. This fusion event provides a possible target for treatment and/or prevention of tumor progression, viral pathogenesis and neurodegenerative diseases where EVs have been implicated as key mediators^{2, 4-6, 65}. Blocking EV cargo release by manipulating the EV-endosome membrane fusion process may assist in preventing or decelerating disease pathogenesis. However, a safe disposal of the EVs e.g. through lysosomal degradation is required in addition to the prevention of intracellular EV cargo release. For instance, treatment of cells with U18666A which prevents exosome-endosome membrane fusion, and consequently intracellular cargo release, has been shown to stimulate exosome secretion by cells⁶⁵. This would unwantedly increase the risk of spreading the disease-causing entities. Indeed, inhibition of exosome secretion by bacteria-infected macrophages was shown to inhibit sepsis-induced inflammation and cardiac dysfunction⁶⁶. Therefore, prevention of exosome secretion by 'diseased' cells and/or inhibition of exosome internalization by surrounding cells may offer safer alternatives to inhibition of EV cargo release in order to prevent EV-mediated spreading of disease.

Acknowledgment

ISZ is supported by the Dutch Technology Foundation TTW, which is part of the Netherlands Organization for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs. Part of the work was funded by The Netherlands organization for scientific research (STW Microscopy Valley 12718, NWO 175-010-2009-023 and ZonMW 91111.006) to BNGG, and de Cock-Hadders Stichting to MAdB and BSJ. BSJ received a NAMASTE scholarship funded by the Erasmus Mundus India-EU mobility consortium.

Competing interest

The authors declare no competing interests.

Author contributions

BSJ and ISZ conceived the project. BSJ and MAdB performed the experiments. All authors developed experimental design, performed analysis, and wrote and edited the manuscript.

Methods

Plasmids

N-Flag-Apex2-emGFP-CD63 (A gift from Nicole C. Meisner-Kober)²¹ was amplified adding BsmBI site at both the ends of the sequence. Next, the amplified segment was inserted into an entry vector pENTR1A (a gift from Eric Campeau and Paul Kaufman; Addgene, plasmid# 17398; <http://n2t.net/addgene:17398>; PRID:Addgene_17398)⁶⁷ by golden gate assembly method⁶⁸. The plasmid obtained was then recombined with pLenti-CMV-Puro-DEST (a gift from Eric Campeau and Paul Kaufman; Addgene, plasmid# 17452; <http://n2t.net/addgene:17452>; PRID:Addgene_17452)⁶⁷ using a gateway LR clonase enzyme (ThermoFisher Scientific, 11791100) to achieve the expression vector pLenti-CMV-N-Flag-APEX2-emGFP-CD63-Puro-DEST. For GFP display at the surface of EVs, emGFP was inserted at the second extracellular loop of CD63 following a reported strategy, to generate pLenti-CMV-N-CD63-emGFP-CD63-C-Puro-DEST⁶⁹. To generate the expression vector pLenti-CMV-CD63-mRFP using the same cloning strategy, CD63 was amplified from N-Flag-Apex2-emGFP-CD63 and mRFP sequence was amplified from Lamp1-mRFP plasmid (a gift from Walther Mothes; Addgene, plasmid# 1817; <http://n2t.net/addgene:1817>; PRID:Addgene_1817)⁷⁰. mAzami-Green (mAG)-galectin 3 (Gal3) plasmid was a gift from Niels Geijsen (Addgene, plasmid# 62734; <http://n2t.net/addgene:62734>; PRID:Addgene_62734)⁷¹. The secretion signal peptide sequence in the mCherry-APEX2-anti-GFP FLIPPER-body vector⁷² was removed during PCR and pLenti-CMV-mCherry-APEX2-anti-GFP FLIPPER-body-Puro-DEST was generated using aforementioned golden gate and gateway cloning strategy. In this study, the APEX2 in the FLIPPER-body was not used, and therefore the probe is called fluobody in the text. Full sequences of used proteins are provided in the supplementary data.

Generation of EV producer and acceptor HEK293T cell lines and cell culture

The EV producer cell lines (GFP-CD63 and CD63-RFP) and acceptor cell lines (mCherry anti-GFP fluobody and galectin-3 Azami-Green) were created via lentiviral transduction followed by Puromycin (Sigma P8833) selection at 1 μ g/ml. HEK293T cells were cultured in DMEM (Gibco 41965-039) supplemented with 10 % Fetal Bovine Serum (FBS, Bodinco, 5010) and 1 % Penicillin-Streptomycin sulfate (Gibco, 15140-122) at 37 °C under 5 % CO₂. For LM imaging, acceptor cells were seeded on glass cover slips coated (VWR, 631-0150) with poly L-Lysine (Sigma, P-2636). For CLEM imaging, acceptor cells were seeded on glass bottom Petri dishes (Greiner, 627870). The Petri dishes were, before cell seeding, sputter coated with 2 nm palladium/gold (LeicaSCD050) after which a pattern was made to enable to relocate the ROI in EM as defined by LM.

Preparation of exosome-depleted medium

DMEM containing 10 % FBS was centrifuged at 110,000 $\times$ g for 16 h at 4 °C to deplete the fetal bovine serum-derived EVs. The resulting supernatant was filter sterilized through a 0.2- μ m filter (Millipore) and stored at 4 °C.

EV isolation

EV-producer cells were seeded in T162 flasks (Corning), and 15 ml exosome-depleted medium was added when cells reached ~40 % confluence. 48 hours later medium was collected and

EVs were isolated by sequential centrifugation (**Table S1**). Please note that the EV fraction obtained after ultracentrifugation at 100,000g represents small EVs. The final pellet was resuspended in 50 μ L PBS and protein concentration was measured with DC protein assay kit (Bio-Rad, 5000114). EV concentrations are given as total EV protein weight per volume (μ g/ml). Of note, besides EVs, other proteinaceous medium components may have become pelleted by sequential centrifugation, which may have caused an overestimation of the EV protein content.

SDS-PAGE and western blot

SDS-PAGE samples were prepared using an established protocol⁷³. Briefly, after measuring the protein content of EVs and cell lysates, 30 μ g protein per sample was mixed with Laemmli loading buffer with SDS and protease inhibitors (Roche, 11697498001). Samples were boiled for 5 minutes at 90 °C, loaded on a 10 % SDS-PAGE gel and subjected to electrophoresis at 100V for 2 hours. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, IPFL00010) and blocked with Odyssey blocking buffer (Li-COR, 927-40000) for 1 hour at RT. Blots were incubated overnight with primary antibodies (**Table S2**) in blocking buffer at 4 °C. Next, blots were washed with 0.1 % PBS-Tween20 and subsequently incubated in secondary antibody for 1 hour at RT. They were washed with 0.1 % PBS-Tween20 and imaged with an Odyssey Infrared Imaging system (Li-COR).

Size, polydispersity, and ζ -potential of EVs

An EV suspension with a concentration of 10 μ g/ml in PBS was loaded into a folded capillary cell (Malvern Instruments DTS1070) and measured on a Zetasizer Nano ZS particle analyzer (Malvern Instruments) using a 633 nm laser. Dynamic light scattering measurements were performed in triplicate. Size and polydispersity were calculated by the cumulant analysis method using Zetasizer software version 7.10. The ζ -potential was determined by measuring the electrophoretic mobility and calculated using the Smoluchowski approximation.

mAG-gal3 endosomal permeabilization assay

mAG-gal3 expressing HEK293T cells were seeded in 24 well plates on glass coverslips at a density of 150×10^3 cells/ml (0.5 ml) and treated the next day with 0.5 ml of 20 μ g/ml CD63-RFP EVs for 2, 4, 8 or 12 hours in exosome-depleted medium. Lipoplex-treatment was used as a positive control. Lipoplexes composed of Lipofectamine 2000 and pDNA were prepared according to the manufacturer's instructions and incubated with mAG-gal3 cells for 12 hours in exosome-depleted medium. Control cells were treated in exosome-depleted medium in the absence of CD63-RFP EVs. The effect of a high(er) concentration of CD63-RFP EVs on endosomal integrity in mAG-Gal3 expressing cells was studied by using 0.5 ml of 100 μ g/ml EVs, while keeping the rest of the protocol unchanged. Cells were fixed (4% PFA) and examined for mAG-gal3 punctae formation by fluorescence microscopy (Leica DMI6000B fluorescence microscope (HCX PL FLUOTAR 63x/ N.A. 1.25 OIL).

Anti-GFP fluobody EV cargo release assay

HEK293T cells expressing mCherry anti-GFP fluobody were seeded in 24 well plates on glass coverslips at a density of 150×10^3 cells/ml (0.5 ml). The next day, cells were treated with 0.5 ml of 20 μ g/ml GFP-CD63 EVs for 2, 4, 8 or 12 hours in exosome-depleted medium. Control

cells were treated in exosome-depleted medium in the absence of GFP-CD63 EVs. Cells were fixed and mCherry fluobody punctae formation was examined by fluorescence microscopy.

Pharmacological inhibitor, temperature, and fluorescent tracer treatments of HEK293T cells

HEK293T cells expressing mCherry anti-GFP fluobody were plated in 24 well plates on glass coverslips at a density of 150×10^3 cells/ml (0.5 ml). The next day, cells were treated with 0.1 μ M Bafilomycin A1 (BafA1, Enzo Lifesciences BML-CM110-0100) for 30 minutes in 0.5 ml exosome-depleted medium, followed by addition of 20 μ g/ml (10 μ g per well) EVs in continued presence of the drug for 12 hours. Cells were treated with 1 μ g/ml U18666A (Merck Millipore, 662015-10MG) for 4 hours in 0.5 ml of exosome-depleted medium, followed by addition of 20 μ g/ml (0.5 ml) EVs in continued presence of the drug for 12 hours. LysoTracker Red DND-99 (Invitrogen, L7528) was added cells in the inhibitor experiments 1 hour before fixation. TopFluor (Bodipy)-cholesterol (Avanti lipids, 810255P) was added (10 μ M) to the cells for 1 hour before addition of inhibitors. Cells were (pre)treated with 80 μ M dynasore (Bioconnect, 2897/10) for 30 min in 0.5 ml of exosome-depleted medium, followed by addition of 20 μ g/ml (0.5 ml) EVs in continued presence of the drug for 2 hours. To inhibit energy dependent passage across the plasma membrane, cells were incubated at 4 °C for 30 min and then treated with 0.5 ml of 20 μ g/ml GFP-CD63 EVs in ice-cold EV-depleted medium for 1 hour before fixation. Control cells were treated similarly at 37 °C before fixation.

MTT Assay

The viability of HEK293T after exposure to pharmacological inhibitors was evaluated by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich #M2128) assay. 2×10^4 (0.2 ml) HEK293T cells were seeded in 96-wells plates precoated with PLL. Cells were treated in triplicate with 0.1 μ M BafA1 and 1 μ g/ml U18666A in exosome depleted DMEM (final volume of (0.2 ml), for 21 hours. Untreated cells in exosome depleted DMEM were used as a negative control. During the final 3 hours of incubation, cells were exposed to 20 μ l MTT solution (5 mg/ml in PBS). The medium was removed and formazan crystals were dissolved in 200 μ l of DMSO. Upon complete solubilization of the crystals, the optical density of each well was measured at a wavelength of 520 nm using a microplate spectrophotometer (μ Quant, BIO-TEK Instruments inc).

Electron microscopic investigation of isolated EVs

Isolated EVs were fixed in 50 μ l 2 % paraformaldehyde (PFA, Merck, 1.04005.1000) prepared in 0.1 M sodium cacodylate buffer pH 7.4 (Sigma, C0250-500g). 4 μ l of the EV solution was incubated on formvar-coated 150 meshed copper grids (Electron Microscopy Sciences, 0150-Cu) for 25 minutes. The grids were rinsed with PBS for 1 minute and subsequently incubated with 1 % glutaraldehyde (GA, Polysciences, 01909-100) in 0.1 M sodium cacodylate buffer pH 7.4 for 5 min followed by rinsing with MilliQ 7 times. All steps were performed at RT. For EV immunostaining, grids were incubated for 1 hour with primary anti-GFP antibody, rinsed and incubated for 1 hour with a secondary antibody conjugated to 10 nm gold. Next, grids were incubated with 2% uranyl oxalate (pH7; SPI, 02624-AB) for 4 minutes on ice, briefly rinsed and incubated for 10 minutes in methyl cellulose-uranyl acetate (pH 4) on ice. Images were generated by EM (FEI, CM100).

Fixation and immunolabeling for LM

Cells were fixed for 30 minutes with 4 % PFA in PBS and rinsed with PBS. Prior to immunolabeling, cells were permeabilized (0.2 % Tween-20 (Sigma, P1379) in PBS) and treated with blocking solution (1 hour; 3 % BSA (Sigma, A7906) in PBS). Cells were then incubated with primary antibodies overnight at 4 °C (**Table S2**) in blocking solution followed by rinsing with PBS. Next, cells were incubated with secondary antibodies in blocking solution and subsequently rinsed with PBS. DAPI (1 µg/ml in PBS; Sigma, D9542) was added for 20 minutes, followed by rinsing with PBS and mounting of the coverslips using Vectashield (Vector Laboratories, H-1000) onto microscope slides. Images were generated using confocal microscopy (Leica SP8, HC PL APO CS2 63x/ N.A. 1.4). All steps were performed at RT unless mentioned otherwise.

Fixation and imaging for CLEM

An equal volume of 2 % PFA and 0.2 % GA in 0.1M sodium cacodylate buffer (pH 7.4) was added to the cells in medium and incubated for 10 minutes. Cells were then incubated with fresh pure fixative (2 % PFA and 0.2% GA) for 30 minutes and rinsed twice with 0.1 M sodium cacodylate. All steps were performed at RT. Fluorescent images were generated using confocal microscopy (Zeiss LSM 780, Plan-Neofluar 63x/ N.A. 1.3 Imm Corr DIC M27 lens) before being processing for EM.

Embedding and EM

Epon EM embedding was performed as described previously⁷⁴. The ROI identified by confocal microscopy was traced using a stereo microscope by using the palladium/gold marks. The selected areas were sawn out and trimmed prior to ultrathin sectioning. Serial sections (100 nm) of entire cells were collected on nickel one-hole grids (Electron Microscopy Sciences, 1000-Ni). For stabilization, the grids were pre-irradiated using a TEM (FEI, CM100) at 80 KV. Subsequently, large-scale areas, with 2.5 nm pixel size, were scanned using the STEM detector in a SEM (Zeiss Supra55) at 28 KV^{39, 74}. An overlay was made in Adobe Photoshop. All datasets are available at www.nanotomey.org.

Post-embedding immuno-labeling

Thin sections in epon were etched for 10 minutes in 1% periodic acid (Merck, 1.00524.0025) in MilliQ, followed by washes (3x2 minutes MilliQ) and blocking with 1% BSA in PBS for 30 minutes. Next, the sections were immunolabeled (anti-GFP, Table S2) for 4 hours, washed 2x5 min with PBS and incubated with a biotinylated secondary antibody (1 hour). Samples were rinsed (3x5 minutes PBS) and incubated with Quantum dot 655 (QD655)-labeled streptavidin for 1 hour. Finally, sections were rinsed (3x5 minutes PBS). Images were generated with 2.5 nm pixel size, using the STEM detector in a SEM (Zeiss Supra55) at 28 KV.

LM image analysis

Representative images were selected and cropped. The images were adjusted in Adobe Photoshop by adding a layer to change the levels, which was implemented to all the images to the same extent. Quantification analysis was performed on the recorded z-stacks and analyzed using the software Icy⁷⁵. The Colocalizer with binary and excel output plugin was used to determine the number of EVs and fluobody spots, and colocalization.

For colocalization studies in cells coincubated with GFP-CD63 and CD63-RFP EVs, images were first adjusted in brightness/contrast minimum and maximum before colocalization measurements were applied. Brightness/contrast minimum and maximum were set at 2-70 for GFP-CD63, and 1-35 for CD63-RFP⁷⁶. Next, Fiji plugin colocalization threshold was used, without the use of a ROI, to obtain the Pearson's value for the whole image. For colocalization studies in immunostained fluobody-expressing cells, fluobody (red channel) adjustments were set at 11 (min) and 84 (max). Similarly, for LAMP1 (633 channel) brightness/contrast minimum-maximum was set at 0-70. JACoP plugin was used to obtain the Mander's coefficient⁷⁶. Values were calculated after setting a threshold to remove the background to only include fluobody punctae (mCherry channel) or LAMP1 (633 channel) spots.

Live cell imaging of mAG-gal3 HEK293T cells

For live cell imaging, cells were grown on glass bottomed 2 wells plates (Lab-TEK chambered coverglass, Thermo Fisher Scientific, Denmark). On the day of the experiment, cells were placed in a DeltaVision Elite microscope equipped with a temperature/CO₂ controlled cabinet and automated stage. HEK293T cells expressing mAG-gal3 were selected before addition of CD63-RFP EVs. Experiments were carried out in exosome-depleted medium. Image acquisition took place from 30-270 min after the addition of the CD63-RFP EVs to the cells using softWoRx 6 acquisition and integrated deconvolution software (GE Healthcare, Issaquah, WA). The UPLSAPO objective (100x Oil, NA 1.4, WD 0.12mm) and GFP/mCherry filter combination were used for image acquisition. Images were further analyzed using Fiji⁷⁷.

Statistical analysis

Experiments were performed at n=3. Data are presented as mean $\pm$ SD. The significance of the difference between two independent samples was determined using Student's t-test. Groups were compared using one-way analysis of variance, with Tukey's post hoc. All the statistical analyses were performed using GraphPad Prism.

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Supplementary information

Figure S1 | Biophysical characterization of WT EVs and GFP-CD63 EVs isolated from HEK293T cells and GFP-CD63 HEK293T cells, respectively. (A) HEK293T cells expressing GFP-CD63. Note the punctate staining pattern, resembling endosomal localization. Bars: 10 μm . (B) Top: TEM of purified WT and GFP-CD63 EVs derived from HEK293T cells. Bottom: higher magnification of boxed areas. Bars: top 2 μm , bottom 0.1 μm . (C) Western blot analysis of lysates of WT and GFP-CD63 HEK293T producer cells and isolated EVs (30 μg protein per lane). GFP is only present in GFP-CD63 cells and EVs. EV marker TSG101 is enriched in both WT and GFP-CD63 EVs relative to cells. EV markers CD9 and LAMP1 are present in both EV types. EV-negative marker golgin-97 is depleted from both EV types relative to cells. β -actin: loading control for cell lysates. EV: EV lysate; Cell: whole cell lysate. (D) Size distribution by intensity of purified WT and GFP-CD63 EVs derived from HEK293T determined by Malvern zeta sizer. WT and GFP-CD63 EVs have an average diameter of 159.7 ± 3.4 nm and 163 ± 0.9 nm and polydispersity index of 0.264 ± 0.021 and 0.276 ± 0.027 , respectively. (E) Size distribution by number of purified WT and GFP-CD63 EVs derived from HEK293T determined by Malvern zeta sizer. (F) Zeta potential (surface charge) profiles of purified WT and GFP-CD63 EVs determined by Malvern zeta sizer. GFP-CD63 overexpression does not alter size and surface charge of EVs.

Figure S2 | GFP-CD63 EVs are taken up via endocytosis. (A) HEK293T cells were incubated with GFP-CD63 EVs in the absence and presence of dynasore (dynamin inhibitor) for 2 hours. Note the absence of EVs in dynasore-treated cells, indicating a role for dynamin-dependent endocytosis in EV uptake. Bars: 10 μ m. (B) HEK293T cells were incubated with GFP-CD63 EVs at 37 $^{\circ}$ C and 4 $^{\circ}$ C for 1 hour. Note the absence of EV uptake at 4 $^{\circ}$ C, indicating a role for active transport in EV uptake. GFP: EV; Blue: nucleus. Bars: 10 μ m.

Figure S3 | EVs localize in membrane-bound compartments in HEK293T acceptor cells. The underlying structures of GFP-positive spots, representing internalized GFP-CD63 EVs in HEK293T cells following (A) 4 hours and (B) 12 hours of incubation, are membrane-bound compartments, as revealed by CLEM. Bars: CLEM 5 μ m, EM 0.2 μ m. Full data set is available via www.nanotomy.org.

Figure S4 | GFP-CD63 and CD63-RFP EVs show similar EV characteristics and intracellular localization in recipient cells. **(A)** Top: GFP-CD63 and CD63-RFP HEK293T cells. Note the similar fluorescence localization pattern in both producer cell lines. Bottom: Cartoon of GFP-CD63 and CD63-RFP fusion proteins and EVs displaying the respective fluorescent protein inside the EV. Bars: 10 μ m. **(B)** Western blot analysis of lysates of GFP-CD63 and CD63-RFP HEK293T producer cells and derived EVs (30 μ g protein per lane). Note that GFP and RFP are exclusively present in corresponding cell lines and derived EVs, i.e. GFP-CD63 and CD63-RFP cells and EVs, respectively. EV marker TSG101 is enriched in EVs compared to producer cells. β -actin: loading control for cell lysates. **(C)** Cells were incubated for 12 hours with equal amounts of GFP-CD63 and CD63-RFP EVs simultaneously and imaged following fixation. Note partial colocalization (yellow dots, Pearson's Colocalization Coefficient = 0.37), showing the presence of both EV types in the same endocytic compartments. This confirmed that GFP-CD63 and CD63-RFP EVs show similar EV characteristics and intracellular localization in recipient cells, and were fit to be used interchangeably. Green, GFP-CD63 EV; red, CD63-RFP EV; blue, nucleus. (Scale bars, 10 μ m.) **(D)** GFP fused to the N-terminus of CD63 is located at the inside of GFP-CD63 EVs. Wildtype EVs, GFP-CD63 EVs (GFP at N-terminus of CD63), and outGFP-CD63 EVs (GFP in the loop of CD63) were labeled, without prior permeabilization, with anti-GFP antibody and secondary antibody conjugated to gold, and investigated by TEM. Only outGFP-CD63 EVs show gold-labeling. Bars: 50nm.

Figure S5 | CD63-RFP EVs do not induce endosomal permeabilization. Fluorescence images of mAG-gal3 expressing HEK293T cells incubated with 20 $\mu\text{g}/\text{ml}$ CD63-RFP EVs (EV) for 2, 4, 8 and 12 hours. Cells were fixed before imaging. DAPI: Nucleus; RFP: EVs; mAG: Gal3. Bars: 10 μm .

Figure S6 | Increased EV concentration does not induce endosomal permeabilization. Fluorescent images of mAG-Gal3 expressing HEK293T cells incubated with 100 µg/ml CD63-RFP EVs for 2, 4, 8 and 12 hours. Cells were fixed before imaging. Note the absence of mAG-gal3 punctae at all investigated time points, similar to in control (untreated) cells. DAPI: Nucleus; mAG: Gal3; RFP: EVs. Bars: 20 µm.

Figure S7 | EVs do not induce endosomal permeabilization. Still images of Movie 1. Live-cell imaging of mAG-gal3 HEK293T cells incubated with 20 µg/ml CD63-RFP EVs. Time (min). RFP: EV; mAG: Gal3. Bar: 5 µm.

Movie 1 | EVs do not induce endosomal permeabilization. Of note, chemical fixatives may induce protein extraction or relocalization in cells¹. To substantiate the absence of mAG-Gal3 punctae formation upon incubation of CD63-RFP EVs with mAG-gal3 HEK293T cells, the experiment was performed on live cells. mAG-gal3 HEK293T cells were incubated with 20 µg/ml CD63-RFP EVs for 4 hr and followed by live cell imaging. Following uptake of EVs by mAG-gal3 HEK293T cells, as revealed by the appearance of red fluorescent punctae in the cell cytosol, mAG-gal3 punctae formation was not observed. Still images in Figure S7.

Figure S8 | Quantification of fraction of mCherry-fluobody-expressing cells that show fluobody punctae after 2, 4, 8, and 12 hours incubation with 20 µg/ml CD63-GFP EVs. Note that fluobody punctae are seen in 94% ± 4.4 of the cells at the 12-hour time point (>24 cells/condition, error bars represent SD, n=3).

Figure S9 | WT EVs do not induce fluobody punctae in anti-GFP fluobody (mCherry) HEK293T cells. Fluorescence images of HEK293T cells expressing anti-GFP fluobody (mCherry) incubated with HEK293T WT EVs for 4, 8 and 12 hours. Cells were fixed before imaging. Note the absence of fluobody punctae at all investigated time points, similar to in control (untreated) cells. DAPI: nucleus; mCherry: fluobody; GFP: EV. Bars: 10 μ m.

Figure S10 | Effects of BafA1 and U18666A metabolic inhibitors on HEK293T cells (A) MTT cell viability assay of HEK293T cells with and without metabolic inhibitors BafA1 and U18666A (error bars represent SD, n=3; ns, not significant, two-tailed Student's t-test). Cell viability is not affected upon inhibitor treatments. (B) GFP-CD63 EV internalization (t = 12 h) in HEK293T cells in absence and presence of metabolic inhibitors. Number of EV-positive spots in control cells is set at 100%. (3-6 imaging fields and >8 cells/experiment, error bars represent SD, n=3; ns, not significant, two-tailed Student's t-test). Internalization of EVs is not affected upon inhibitor treatments. (C) Lysotracker staining of cells with and without inhibitor treatments. Note the decrease in lysotracker fluorescence in BafA1 treated cells, indicating neutralization of endosomal pH. (Scale bars, 10 μ m.) (D) Late endosomal marker LAMP1 staining of cells with and without inhibitor treatments. U18666A and BafA1 treatment led to enlarged LAMP1-positive structures. (Scale bars, 10 μ m.) (E) Incubation of cells with Bodipy-cholesterol showed an endosomal staining pattern with increased intensity, indicating cholesterol accumulation in endosomes, in U18666A-treated cells compared to control and BafA1-treated cells. Bars: 10 μ m.

Table S1 | EV isolation method by means of sequential centrifugation

Step	Time (min)	Centrifugal force (g)	Description	Collect	Centrifugation, rotor
1	10	300	Cell removal	Supernatant	Beckman Coulter, Allegra A-15R
2	10	2,000	Remove cellular debris	Supernatant	Beckman Coulter, Allegra A-15R
3	30	10,000	Remove apoptotic vesicles and microvesicles	Supernatant	Sorvall Discovery 90SE, Beckman SW32i
4	70	100,000	Collect EVs	Pellet	Sorvall Discovery 90SE, Beckman SW32i
5	70	100,000	Wash EVs with 5 ml PBS	Pellet	Sorvall Discovery 90SE, Beckman SW32i

Table S2 | List of antibodies used in the study

Technique	Target	Host	Brand	Dilution
Western blot, primary	Golgin-97	Mouse	Invitrogen, A21270	1:1000
	LAMP1	Mouse	DSHB, AB-2296838	1:1000
	GFP	Goat	Rockland, 600-101-275	1:1000
	RFP/mCherry	Rabbit	Abcam, ab167453	1:1000
	TSG101	Mouse	Genetex, GTX70255	1:1000
	CD9	Rabbit	Abcam, ab92726	1:1000
	β -actin	Rabbit	Abcam, ab8227	1:2000
Western blot, secondary	Mouse IgG (H+L)	Goat	Li-COR Bioscience, LI 926-68070	1:5000
	Rabbit IgG (H+L)	Goat	Li-COR Bioscience, LI 926-32211	1:5000
	Goat IgG (H+L)	Donkey	Li-COR Bioscience, LI926-68074	1:5000
FM, primary	LAMP1	Mouse	DSHB, AB-2296838	1:200
FM, secondary	Mouse IgG (H+L), AlexaFluor 633	Goat	Invitrogen, A-21050	1:500
EM, primary	GFP	Goat	Rockland, 600-101-275	1:100
EM, secondary	Rabbit, biotinylated	Goat	DAKO, E0432	1:200
	Streptavidin, QD655		Life Technologies, Q10121MP	1:1000
	Rabbit IgG, 10 nm gold	Goat	BBI solutions, EM.GAR10	1:50

Supplementary data: Nucleotide and corresponding amino acid sequence of cDNAs used in this study

List of marker proteins used in this study, including abbreviations, and references.

Name	Abbreviation	Reference
Monomeric Red Fluorescent protein	mRFP	2, 3
Emerald Green Fluorescent protein	emGFP	4, 5
Monomeric Azami-Green	mAG	6, 7
mCherry	mCh	8
Ascorbate peroxidase 2	APEX2	5, 9
Human Galectin-3	Gal3	7
Tetraspanin CD63	CD63	5
Nanobody anti-GFP	NB	10

Sequence GFP-CD63

APEX2-EmGFP-CD63

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aaggcgaagaagaagctcagaggcttcacgtcgctgagaagagatgcgctcctctaagtctcgtttggcattccac
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tctgctggaacctttgacaagggcacgaagaccggtggacccttcggaaccatcaagcacctgccaactggct
S A G T F D K G T K T G G P F G T I K H P A E L A
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H S A N N G L D I A V R L L E P L K A E F P I L S
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Y A D F Y Q L A G V V A V E V T G G P K V P F H F
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G R E D K P E P P P E G R L P D P T K G S D H L R
gatgtgtttggcaaagctatggggcttactgaccaagatatcgttgctctatctggtgggtcacactattggagct
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gcacacaaggagcgcttctggatttgagggtcacctggacctctaatacctcttattttcgacaactcatacttcacg
A H K E R S G F E G P W T S N P L I F D N S Y F T
gagttgttgagtgtgtgagaaggaaggtctccttcagctaccttctgacaaggctcttttgtctgacctgtattc
E L L S G E K E G L L Q L P S D K A L L S D P V F

cgccctctcgttgacaaatatgcagcggacgaagatgccttctttgctgattacgctgaggtcaccaaaagctt
R P L V D K Y A A D E D A F F A D Y A E A H Q K L
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K F S V S G E G E G D A T Y G K L T L K F I C T T
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V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y
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L M I T F A I F L S L I M L V E V A A A I A G Y V
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F R D K V M S E F N N N F R Q Q M E N Y P K N N H
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T A S I L D R M Q A D F K C C G A A N Y T D W E K
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R S G Y E V M *

Sequence outGFP-CD63

N-CD63-GFP-CD63-C

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 C A C A V G L I A V G V G A Q L V L S Q T I I Q G
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 C C G A C K E N Y C L M I T F A I F L S L I M L V
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 I C T T G K L P V P W P T L V T T F T Y G V Q C E
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 R I E L K G I D F K E D G N I L G H K L E Y N Y N
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 S H K V Y I T A D K Q K N G I K V N F K T R H N I
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 S G Y E V M *

Sequence CD63-mRFP

CD63-mRFP

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V G L I A I G V A V Q V V L K Q A I T H E T T A G
tcgctgttgccctgtggtcatcattgcagtggggtgccttcctctcctggtggcctttgtgggctgctgtggggcc
S L L P V V I I A V G A F L F L V A F V G C C G A
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C K E N Y C L M I T F A I F L S L I M L V E V A V
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A I A G Y V F R D Q V K S E F N K S F Q Q Q M Q N
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Y L K D N K T A T I L D K L Q K E N N C C G A S N
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P F A W D I L S P Q F Q Y G S K A Y V K H P A D I
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G A L K G E I K M R L K L K D G G H Y D A E V K T
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T Y M A K K P V Q L P G A Y K T D I K L D I T S H
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Sequence mAG-Galectin-3

mAG-Gal3

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I E G E G K G N P Y E G T Q I L D L N V T E G A P
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L P F A Y D I L T T V F Q Y G N R A F T K Y P A D
atccaggactacttcaagcagaccttccccgaggggtaccactgggagaggagcatgacctacgaggaccagggc
I Q D Y F K Q T F P E G Y H W E R S M T Y E D Q G
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I C T A T S N I S M R G D C F F Y D I R F D G T N
ttccccccaacggccccgtgatgcagaagaagaccctgaagtgggagccagcaccgagaagatgtacgtggag
F P P N G P V M Q K K T L K W E P S T E K M Y V E
gacggcgtgctgaagggcgacgtgaacatgaggtgctgctgtagggggcgccgactacaggtgcgacttcaag
D G V L K G D V N M R L L L E G G G H Y R C D F K
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T T Y K A K K E V R L P D A H K I D H R I E I L K
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P G Q A P P G A Y P G Q A P P G A Y P G A P G A Y
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P G A P A P G V Y P G P P S G P G A Y P S S G Q P
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P L P G G V V P R M L I T I L G T V K P N A N R I
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K P F K I Q V L V E P D H F K V A V N D A H L L Q
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S Y T M I *

Sequence Fluobody

mCh-APEX2-Nanonody

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S V N G H E F E I E G E G E G R P Y E G T Q T A K
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L K V T K G G P L P F A W D I L S P Q F M Y G S K
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A Y V K H P A D I P D Y L K L S F P E G F K W E R
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V M N F E D G G V V T V T Q D S S L Q D G E F I Y
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K V K L R G T N F P S D G P V M Q K K T M G W E A
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S S E R M Y P E D G A L K G E I K Q R L K L K D G
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G H Y D A E V K T T Y K A K K P V Q L P G A Y N V
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L A F H S A G T F D K G T K T G G P F G T I K H P
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A E L A H S A N N G L D I A V R L L E P L K A E F
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P I L S Y A D F Y Q L A G V V A V E V T G G P K V
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P F H P G R E D K P E P P P E G R L P D P T K G S
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D P V F R P L V D K Y A A D E D A F F A D Y A E A
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H Q K L S E L G F A D A A A A M D Q V Q L V E S G

Chapter 4

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M R W Y R Q A P G K E R E W V A G M S S A G D R S
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S Y E D S V K G R F T I S R D D A R N T V Y L Q M
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N S L K P E D T A V Y Y C N V N V G F E Y W G Q G
accaggtcaccgtctccagctaa
T Q V T V S S *
```

References supplementary data

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Chapter 5

Lanthanide-conjugated antibodies do not allow EDX-based protein detection in electron microscopy

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Abstract

Electron microscopy (EM) provides protein visualization at high resolution within the ultrastructural context and imaging is based on (added) electron density of biological material. Addition of heavy-metal probes such as gold nanoparticle conjugated to immunoglobulin type G (IgG) antibodies adds electron density to the protein of interest within ~25 nm. However, labeling multiple proteins in EM is limited due to the grey-scale based imaging and the low number of different sized nanoparticles that can be used. To expand the number of targets, energy dispersive X-ray analysis (EDX) can be used as additional information to examine the elemental composition of the specimens in EM. Using a new-generation of EDX detectors, either alone or in arrays, the level of sensitivity allows more informative EDX imaging of biospecimens. We address here whether IgG coupled to lanthanides, that are being used for protein identification in e.g. time-of-flight mass cytometry, can also be used to identify protein targets by mapping the lanthanides in EM. As a proof-of-concept, secondary anti-mouse or anti-rabbit IgGs were conjugated to polymers with neodymium (Nd) or terbium (Tb), respectively. When the conjugated IgGs were analyzed, the Nd and Tb were both detected in EDX analysis. However, when used in a cellular labeling, neither the Nd nor Tb exceeded the background, rendering them undetectable. This indicates that the IgGs have too few atoms to produce enough X-rays to be detected, and hence an increase in the number of atoms per IgG is required. Alternatively, the sensitivity of the current X-ray detectors is not sufficient for our attempted approach, capturing only a small fraction of all the X-rays produced. Currently, the IgGs coupled to lanthanides are not suitable for EDX analysis in life sciences.

Keywords: Probes, Lanthanide antibodies, EDX, ColorEM, Electron microscopy

Introduction

Protein identification at high resolution is desirable to gain additional insight in the localization and function of proteins within cells, tissue and the human body. Probes are required to localize specific proteins at high resolution with electron microscopy (EM). EM probes are based on the addition of electron density to the protein, represented as dark marks in the grey-scale image. This grey-scale based imaging limits the ability of multiple protein targets¹. Commonly used probes in genetically-encoded labeling are based on peroxidases^{2,3}, that can precipitate diaminobenzidine (DAB) which is enhanced with osmium (Os). This results in a black staining, but different peroxidases cannot be discriminated, like is done with fluorescent proteins in light microscopy (LM). Another way of protein labeling is affinity-based, in which immunoglobulin type G (IgG) antibodies can be applied before (pre) or after (post) EM embedding. Pre-embedding labeling of intracellular targets require detergents to make the membranes permeable for probe accessibility, by dissolving parts of the membranes⁴. Performing the labeling post-embedding will preserve the ultrastructure, but because of the harsh EM chemicals, epitopes are less available¹. In both labeling strategies, the IgGs are fused with nanoparticles like gold (Au) or quantum dots (QDs)⁵. The Au and QDs nanoparticles differ very little in morphology, but discrimination can be made based on their size and electron density⁶.

The nanoparticles are composed of different elements, which opens the opportunity for imaging based on the elemental composition instead of shape, size or electron density. One technique used in material sciences is energy dispersive X-ray spectroscopy (EDX). In EDX the electron beam (E beam) can dislodge an electron from an atomic shell, leaving an unstable vacancy (Figure 1A). The created vacancy will be filled with an electron from an outer shell, and thereby it will release energy as a characteristic X-ray. The X-rays can be

Figure 1 | Basic principles of X-ray analysis integrated in EM. (A) The electron beam (E beam) can dislodge an electron from e.g. the K shell, after which an unstable vacancy appears. This will be filled with an electron from e.g. the L shell. This gives an atom specific energy release as a characteristic X-ray. **(B)** Schematic representation of the microscope configuration in which transmitted electrons are detector for conventional EM imaging. In addition, X-rays that are produced by the E beam can be collected with an additional detector (the X-ray detector).

detected by adding an X-ray detector to an existing EM system (**Figure 1B**). This allows it to map the elemental composition by collecting the EDX spectra per pixel⁷. The elemental composition of the sample can be represented as a color map and an overlay with the EM image can be made, resulting in 'colorEM'⁷⁻⁹.

Proteins are composed mostly of carbon (C), oxygen (O), nitrogen (N), and may contain phosphorus (P) and sulfur (S). This makes it impossible to discriminate proteins based on relative amounts of elements alone. Using affinity-based probes containing different elements will therefore provide more color to the grey EM image. Previously, it was shown that Au nanoparticles and QDs, with core element cadmium (Cd)⁵, can be discriminated based on their elemental composition⁸, but for multiple target labeling expanding the colorEM toolbox is needed.

To potentially increase the toolbox for immuno-EM, we here consider heavy metal conjugated IgGs that are currently used for time-of-flight mass cytometry (CyTOF)¹⁰⁻¹³, multiplexed ion beam imaging (MIBI)¹⁴⁻¹⁶ and imaging mass cytometry (IMC)¹⁶⁻¹⁹. These techniques allow protein detection for cells in suspension (CyTOF) or for cells in tissue (MIBI and IMC). In these techniques, detection is allowed of >40 IgGs simultaneously. The detection of proteins in these techniques depend on the IgGs and used metal isotope. The IgGs contain approximately 161 rare lanthanide metals per IgG^{10, 20}. In this study, lanthanide conjugated IgGs are examined as a new colorEM probe, in order to combine high resolution EM imaging with multiple protein identification.

Results

To first check if IgGs can be discriminated with EDX based on the conjugated lanthanides, secondary IgGs were conjugated with 161 neodymium (Nd) or terbium (Tb) atoms per IgG.

Figure 2 | EDX detection of lanthanides conjugated to IgGs. EDX spectroscopy of spotted IgG(Nd) (Black) and IgG(Tb) (Red). Expected characteristic X-rays for Nd at 5.23 keV (L shell) and for Tb at 6.27 keV (L shell). Here, both elements show the expected peak.

The generated anti-mouse IgG(Nd) and anti-rabbit IgG(Tb) were spotted and EDX analysis was performed to examine the presence of the lanthanides. IgG(Nd) and IgG(Tb) were detected at the expected L shell peaks of 5.23 keV and 6.27 keV, respectively (**Figure 2**). This shows the presence of the lanthanides on the IgGs and in addition the experiment validates the use of the EDX detector/ EM set-up for Nd and Tb detection.

Adding long polymers with lanthanides to IgGs potentially blocks the antigen recognition site of the IgGs. Therefore, the binding capacity of the IgGs were examined with light microscopy (LM). First, a primary mouse anti-EGFR IgG was applied on epidermal growth factor receptor (EGFR)-GFP producing cells. EGFR is a transmembrane protein mainly localized at the plasma membrane²¹ and the

extracellular domain harbors the epitope recognized by the IgG. The GFP fusion on EGFR is intracellularly so it will not interfere with the epitope recognition. Next, secondary goat anti-mouse IgGs which are conjugated to Alexa Fluor 647 (**Figure 3A**) or QD655, a fluorescent and electron dense nanoparticles (**Figure 3B**). Both fluorescent labels showed colocalization with the EGFR-GFP, confirming the presence of the primary IgG. Finally, the secondary IgG(Nd) binding capacity was validated using an additional third fluorescence donkey anti-goat IgG (**Figure 3C**). These data demonstrate the binding of the primary anti-EGFR IgG as well as the ability for the IgG(Nd) to bind its target without effecting the epitope recognition site due to the added polymer.

Detection limit of Nd in thin EM sections

Probe efficiency for their use in colorEM was determined by using IgGs conjugated with QD655 or IgG(Nd) in immunolabeled cells. Analysis was now performed on EGFR-GFP expressing cells, labeled with a primary anti-EGFR IgG, followed by a secondary IgG conjugated with QDs or Nd. First, the LM image revealed co-localization (yellow) between EGFR-GFP (green) and the labeling with QDs conjugated IgGs (red) (**Figure 4A**). Labeling with QDs was used as positive control for both EM and EDX analysis as it is electron dense and has a Cd core⁵. Correlated light and electron microscopy (CLEM) was used to locate EGFR-GFP positive cells in the 200 nm thick EM section, by using a large-scale nanotomography scan. The zoomed-in area shows the QDs at the plasma membrane (**Figure 4B**; EM). Here, also the

Figure 3 | Lanthanide coupled IgGs binds to the primary anti-EGFR IgGs. EGFR-GFP expressing HEK293T cells are labeled with a primary mouse anti-EGFR IgG. Secondary anti-mouse IgG labeling conjugated with **(A)** Alexa Fluor 647 and **(B)** QD655, validated the binding of the primary IgG. **(C)** Binding of the goat anti-mouse IgG(Nd) was validated using a third donkey anti-goat IgG conjugated to DyLight 549. Bars: 10 μ m.

EDX images of osmium (Os) and Cd are depicted (**Figure 4B**; Cd and Os). Osmium is used in EM sample preparation as second fixative and bind to the lipids in membranes. Note that both Cd and Os are present in at the plasma membrane. The Cd signal of the QDs, L shell at 3.13 keV, was also visible in the EDX spectrum (**Figure 4C**). Thus, QDs have enough Cd for detection with EDX on EM samples in our setup.

The capability of the IgG(Nd) in immunolabeling for colorEM detection in thin EM slices was examined next. CLEM was applied on the cells labeled with secondary IgG(Nd), in order to locate the EGFR-GFP positive cells in large nanotomography scans (**Figure 4D**). The cells were examined with high resolution and did not show any electron dense labels at the zoomed in areas (**Figure 4E**; EM). Using EDX image analysis, Os was found to be present at the plasma membrane (**Figure 4E**; Os). Nd was expected to be present as well at the plasma membrane, but no positive signal was observed (**Figure 4E**; Nd). In addition, the EDX spectrum did not exhibit a peak at the expected energy for Nd, L shell 5.23 keV (**Figure 4F**). A comparison between the EDX spectra of QDs and IgG(Nd) (**Figures 4C, F**) shows that while the Cd signal of QDs is clearly distinguishable above background noise in the QD labeled sample, no Nd peak is observed in both spectra at the expected 5.23 keV. Hence it is concluded that the signal-to-noise ratio was too high for Nd detection in the current used EDX detector/ EM system.

Since X-rays are emitted in all directions, improving the sensitivity (i.e. decreasing the detection limit) can be realized by using multiple X-ray detectors at once. This was achieved by using a transmission EM (TEM) equipped with 4 EDX detectors (SuperX). The same cell of interest as above (**Figure 4D**), was examined (**Figure 4G**; EM). Even though there is Os present in the plasma membrane (**Figure 4G**; Os), the Nd map still did not have a signal at the plasma membrane (**Figure 4G**; Nd). In the EDX spectrum, the number of counts was higher compared to the previously shown spectra due to the increased sensitivity, but still no Nd peak was detected above the background noise at 5.23 keV (**Figure 4H**). Thus, in 200 nm thick sections there are not enough Nd atoms to be detected. Therefore, by expanding the sample thickness, the absolute number of IgG(Nd) will increase. Hence, it is attempted to perform EDX analysis on a bulk SEM sample to analyze the whole cell.

Figure 4 | Neodymium is not detected in thin EM sections. EGFR-GFP expressing HEK293T cells were fixed and labeled with a primary anti-EGFR IgG. **(A)** Secondary labeling with IgG-QD655 shown in correlated microscopy to indicate the cell of interest within LM and EM. LM: Merge of red QD655 and green GFP. **(B)** High magnification EM of area depicted in A. QDs are visible as black dots at the plasma membrane, which is also positive for Osmium (Os) and Cd. **(C)** EDX spectrum of area shown in b. A peak is detected at 3.13 keV, which corresponds to the expected L shell peak of Cd. The other peak in this keV range is from chlorine (Cl) which is found the be increased after chemical fixations⁷. **(D)** Secondary labeling with IgG(Nd) shown with correlated microscopy for selection of the cells of interest. **(E)** Zoom in on the plasma membrane, depicted in D. Membranes are stained positive with Os, but not for Nd. **(F)** EDX spectrum of the total area shown in E. A peak was expected at 5.23 keV, but is absent. **(G)** Zoomed in on the plasma membrane as depicted in D. EDX detected on SuperX system containing four detectors. The Nd does not give a positive signal at the plasma membrane. **(H)** EDX spectrum of the area shown in G. A peak was expected at 5.23 keV, but is absent. Bars: A, D; 20 μ m and B, E, G; 0.5 μ m. Full EM datasets are available at www.nanotomography.org/OA/deBeer2020Thesis/.

Bulk samples do not improve Nd detection

The extracellular domain of EGFR was targeted with the primary anti-EGFR IgGs, which makes it suitable for SEM analysis. The cells were cultured on Pd/Au marks to be able to locate EGFR-GFP positive cells in SEM and to select a cell of interest (**Figure 5A**). The Cd peak is clearly detected, confirming the presence of the QDs in the for SEM labeled samples (**Figure 5B**). In the cells stained with IgG(Nd) (**Figure 5C**), again, no Nd peak was detected in the EDX spectrum (**Figure 5D**), even though the absolute number of counts were a factor 3 to 5 higher compared to the thin sections. In both spectra, potassium (K), calcium (Ca) and titanium (Ti) peaks were present. These background peaks were equal in both spectra and thus the data of QD and IgG(Nd) are comparable. Collectively, these data suggest that

Figure 5 | Neodymium not detected in bulk sample. EGFR-GFP expressing HEK293T cells are fixed and labeled with a primary anti-EGFR IgG. **(A)** Cells of interest were relocated using CLEM in IgG-QD655 labeled cells. **(B)** EDX spectrum of the selected region depicted in A. A peak is present at 3.13 keV, representing the Cd of the QD655. Peaks of Cl, K, Ca and Ti are present. **(C)** Secondary labeling with IgG(Nd) and correlated microscopy for indicating cells of interest. **(D)** EDX spectrum of the selected area shown in C. A peak was expected at 5.23 keV, but is absent. Note that the Cd peak at 3.13 keV is only present in B. Bars: 20 μ m.

even though there is an increased number of IgGs per unit area, there is not enough Nd signal detected to reach detection levels, that might be caused by the effect of EM sample preparation on the IgGs.

Tb not detected in post embedding Tokuyasu labeling

In classic EM embedding, samples are exposed to heavy-metals and dehydration series²². The effect, e.g. lanthanide extraction, of these chemicals on the used IgGs was tested by using a post-embedding labeling method called Tokuyasu^{23, 24}. Here, mouse fibroblasts were infected with Salmonella bacteria followed by chemical fixation and cryo-embedding. Cryo-sectioned cells were labeled using a primary IgG targeting the cell wall protein (CWP). Protein A conjugated with 10nm Au nanoparticles targets the primary IgG (**Figure 6A**; EM), resulting in Au nanoparticles present at the bacterial cell wall. Single nanoparticles were detected as well with EDX in the Au map (**Figure 6A**; Au). This is confirmed in the EDX spectrum (**Figure 6B**), where an Au-specific peak is detected at 2.12 keV. In the samples labeled with secondary IgG(Tb) (**Figure 6C**; EM), Tb was expected at the bacterial cell wall. However, at multiple sides and different magnifications no signal was detected (**Figure 6C**; Tb). In addition, the EDX spectrum did not show a clear Tb peak (**Figure 6D**). There are some additional peaks between 2.0 and 3.0 keV, but these are related to the high uranium (U) concentration. The absence of positive signal with Tokuyasu prepared samples ruled out extraction of lanthanide-conjugated IgGs during EM embedding, suggesting that the concentration of Tb is not high enough for EDX detection.

Discussion and future perspectives

Analysis of grey-scale EM images is limited, when analysis is performed based on researcher's interpretation of the morphology. Probes in EM can be differentiated based on size or shape, but this limit multiple targets labeling up to typically three¹. This can be improved by adding EDX detectors to an existing EM. Different probes can now be differentiated based on the elemental composition. To enlarge this colorEM probe toolbox, lanthanide coupled IgGs, used for CyTOF, MIBI and IMC, were examined to apply in EDX.

QDs and 10 nm Au nanoparticles common used probes in EM, and have been detected by EDX detectors^{7, 8}. Au nanoparticles of 2.5 nm contain at least 500 Au atoms²⁵. In this study, 10 nm nanoparticles were used, which means over >1000s of Au atoms, for each IgG. On the other hand, the IgG(Nd) and IgG(Tb) have only approximately 161 lanthanide atoms per IgG²⁰. So probably, there are not enough X-rays produced per IgG to be visible above the background. Therefore, it must be considered to develop IgGs with an enriched number of lanthanide atoms, by creating longer metal-chelating polymers (currently ~68 lanthanides) or to attach of more polymers (currently ~2.4) per IgG to reach at least 500 atoms per IgG comparable to 2.5 nm Au nanoparticles. These additions however, will increase the probe size, which will cause more steric hindrance and thereby decrease the labeling efficiency²⁶.

EDX detectors have become more sensitive in the recent years⁷, but although the QDs were detected, the Cd in both the map and the spectrum just raise above the background signals, to emphasize the overall sensitivity concern of EDX analysis in life sciences. EDX detectors need further improvements by using e.g. larger detectors, optimize the angle or distance between sample and detector or by adding more detectors. The great advantage of EDX is

the easy implementation and the straightforward analysis, but it is not the most sensitive elemental-guided imaging technique. Other, more sensitive, elemental-guided imaging techniques to consider for colorEM with lanthanide coupled IgGs include electron energy loss spectroscopy (EELS)^{7, 9} and nanoscale-secondary ion mass spectroscopy (NanoSIMS)^{7, 27, 28}. EELS measure the transmitted electrons with a spectrometer to reveal the energy losses specific for each atom. Since it measures the transmitted electrons, samples must be ultrathin, but high resolution and sensitivity can be achieved. The detection of the elements

Figure 6 | Post-embedding Tokuyasu labeling does not result in Tb detection. Salmonella bacteria taken up by cells and labeled, via the Tokuyasu method, with primary anti- cell wall protein (CWP) IgG. EDX detection using SuperX detector. **(A)** Protein A (ProtA) 10nm Au detection of the CWP IgG. In the EM image, black dots representing the Au are present at the bacterial cell wall. The EDX image of Au showed a positive gold signal. Uranium (U) is detected in the bacteria cell wall from the contrasting agent. There is no Tb detected. **(B)** EDX spectrum of A, showed a peak at 2.12 keV, that corresponds with the M shell peak of Au. This confirmed the presence of Au. **(C)** Secondary IgG(Tb) did not result in electron dense signal in the EM image and also in the Tb map there was no signal detected. **(D)** The EDX spectrum of C, does not show a peak at the expected 6.27 keV for Tb. Here, the peak of Au is absent. The U peak is twice as high compared to B, which leads to more visible peaks of the U M shell. This explains the extra peak between 2.0 and 3.0 keV. Bars: 0.1 μ m.

is done by setting a detection window before imaging, and due to the small window, detecting multiple elements at once is limited. NanoSIMS is a destructive method at which a focused ion beam scans and etches the sample, to cause neutral and ionized atoms from the material. These atoms are collected and measured with mass spectrometry. The focused ion beam is not able to create as high resolution images as with an electron beam. Therefore, carry out EM first followed by nanoSIMS, can contribute to a merged high resolution color image. So, EELS and nanoSIMS are both more laborious to implement and analyze, but they have an increased sensitivity compared to EDX analysis. The increased sensitivity can contribute imaging using the lanthanide coupled IgGs as probe for colorEM.

To conclude, EDX is not sensitive enough to detect lanthanide coupled IgGs in biological specimens. Nonetheless, using heavy-metal probes with elemental-guided imaging techniques, will contribute to the ultimate goal for objective data analysis in EM, in which we can determine unbiased fingerprinting of protein localization within ultrastructural context.

Acknowledgment

We acknowledge Jeroen Kuipers for technical assistance and all our lab members for feedback and discussion on the manuscript; financial support enabling technology hotel (ETH) funding ZonMW and by The Netherlands organization for scientific research (NWO 175-010-2009-023; ZonMW 91111.006; STW Microscopy Valley 12718).

Methods

IgG - lanthanide conjugation

Carrier-protein free IgG was conjugated to isotopically enriched lanthanide metals via the MAXPAR conjugation protocol (Fluidigm Sciences). In short, 100 µg of IgGs were diluted in R buffer (Fluidigm) and partially reduced with 4mM tris(2-carboxyethyl)phosphine (TCEP, ThermoFisher Scientific) for 30 min at room temperature (RT). In parallel the X8 polymer (Fluidigm) were loaded with mono-isotopic lanthanide ions in L buffer (Fluidigm) for 30 min at 37 °C. Afterwards the reduced IgG was incubated with the loaded X8 polymer for 90 min at 37 °C. Unbound polymer was washed away by washing in a 50 kDa spin filter (Merck Millipore). The IgG was brought to a concentration of 0.5 mg/ml in phosphate buffered saline (PBS) IgG stabilizer solution (Candor). In this study, goat anti-mouse neodymium (IgG(Nd)) and goat anti-rabbit terbium (IgG(Tb)) were generated (**Table 1**).

Table 1 | IgGs used in this study

IgG: Immunoglobulin G; EGFR: Epidermal Growth Factor Receptor; CWP: cell wall protein *unconjugated

Target	Host	Label	Brand	Concentration (mg/ml)	Dilution (fold)
Mouse IgG	Goat	Nd	* ThermoFisher, A16080	0.5	200
Rabbit IgG	Goat	Tb	* ThermoFisher, A16112	0.5	200
EGFR	Mouse	-	Santa Cruz, sc120	0.2	200
Mouse IgG	Goat	Alexa Fluor 647	Invitrogen, A21236	2	500
Mouse IgG	Goat	QD655	Life Technologies, Q-11021MP	1 µM	500
Goat IgG	Donkey	Dylight 549	Jackson IR, 705-505-147	1.5	500
CWP	Rabbit	-	Gift from Colorado State University	Unknown	1000

Spotting

1 µl (0.5 mg) of IgG(Nd) or IgG(Tb) was spotted on carbon grids with formvar film and air dried.

Cell culture and transfection

HEK293T cells were cultured in DMEM (Gibco, 31966-021), supplemented with 5% Fetal Bovine Serum (FBS, Greiner bio-one, 758093) and 1 % Penicillin-Streptomycin (PAA, P01011-1692), at 37 °C under 5 % CO₂. Cells were transfected with pEGFP-N1 EGFR-GFP (gift from Alexander Sorkin: Addgene plasmid # 32751)²⁹, using 1:4 DNA/polyethylenimine 1 mg/ml (PEI, MW 25,000, Polyscience, 23966). The cells were either seeded in glass bottom petri dishes (Greiner, 627870) for transmission EM (TEM), or on poly L-Lysine (Sigma, P6282-5mg) coated coverslips for scanning EM (SEM). Both were sputter coated, before cell seeding, with 2 nm palladium/gold (LeicaSCD050) after which a pattern was made to enable localization of the region of interest (ROI) in EM, previously established light microscopy (LM).

Fixation

The cells were fixed by adding an equal volume of fixative (2 % paraformaldehyde (PFA, Merck, 1.04005.1000) and 0.2 % glutaraldehyde (GA, Polyscience, 01909-100) in 0.1 M sodium cacodylate buffer (pH 7.4, Sigma, C0250-500g) to the medium and incubating for 10 min. Next, the cells were incubated with fresh fixative as described above for 30 min and rinsed afterwards 2x5 min with PBS (pH 7.4). All steps were performed at RT.

Immunolabeling and confocal microscopy

Cells were labeled for 6 hours at RT with the primary IgG (**Table 1**) and rinsed 3x5 min with PBS. Next, the cells were incubated with the secondary IgGs overnight at 4 °C and rinsed subsequently 3x5 min with PBS. Cases where a third IgG step was applied (overnight at 4 °C and rinsed subsequently 3x5 min with PBS), are indicated in the Figure legends. All IgGs were diluted in 1 % bovine serum albumin (BSA, Roche, 10735086001) in PBS. Fluorescent images were generated using confocal imaging (Zeiss LSM 780, Plan-Apochromat 63x/1.40 Oil DIC), prior to EM preparation.

Transmission electron microscopy

Samples were prepared for TEM as described previously³⁰. The ROI identified by confocal microscopy was traced using a stereo microscope on the palladium/gold marks. The glass bottom was removed using hydrogen fluoride (Sigma, 01066) for ~10 min, after which the selected areas were sawn out and trimmed prior to ultrathin sectioning. 200 nm thick sections were collected on single slot carbon grids (Ted Pella, 16541).

For electron beam stabilization, the grids were pre-irradiated using a TEM (FEI, CM100) at 80 keV. Subsequently, large-scale areas, with 2.5 nm pixel size, were scanned using the STEM detector in a Zeiss Supra55 scanning microscope at 28 keV^{22, 30}. Overlay images of LM and EM were made in Adobe Photoshop. All datasets are available at www.nanotome.org/OA/deBeer2020Thesis/.

Scanning electron microscopy

The cells on coverslips for SEM were incubated for 1 hour at 4 °C with 1% OsO₄ (EMS, 140605) in 0.1 M sodium cacodylate buffer followed by rinsing 5x2 min with MilliQ. The cells were dehydrated through an increasing ethanol series (30%, 50%, 70%) for 15 min each step, followed by three times 100% ethanol for 30 min each. Next, cells were incubated with a 1:1 mixture of 100% ethanol / Tetramethylsilane (TMS, Acros Organic, 138471000). Finally, after 10 min, cells were incubated with pure TMS for 15 min and air dried. Samples were carbon coated (LeicaSCD050) for conductivity before EDX analysis. EM images were taken using the in-lens detection in a Zeiss Supra55 scanning microscopy at 10 keV.

Tokuyasu immunolabeling

Salmonella infected mouse fibroblast (3T3) were fixed, embedded in gelatin, and cryo-sectioned using a Leica FC6 and immunolabeled as described previously²⁴. Samples were trimmed and ultrathin sections of 60 nm thick were cut at 120 °C. Immunogold labeling was performed using a cell wall protein (CWP) IgG. Control samples were labeled with protein-A conjugated to 10nm Au nanoparticles (1:50, EM laboratory, Utrecht University). Lanthanide

samples were labeled with IgG(Tb) with additional protein-G conjugated to 10 nm Au nanoparticles (1:20, Arion). Sections were contrasted with uranium (U) and examined using a FEI Tecnai 12 TEM.

EDX analysis

EDX data were generated using an Oxford Instruments X-Max^N 150 mm² Silicon Drift Detector and AZtecEnergy software (Abingdon, UK) mounted on the Zeiss Supra55 SEM.

For X-Max^N detection, samples at a working distance of 7.4 mm were tilted 25°. EDX images of the regions of interest were performed with a resolution of 1024x1024 pixels. These images were generated by the sum of 2 frames with 500 µs pixel dwell time at 10 keV. Spotted IgGs and bulky samples were generated using 60 µM aperture without high current and thin sections using 120 µM aperture with high current.

When indicated, EDX data were generated using a Super X with Velox software mounted on a Talos TEM. For SuperX detection (4 windowless silicon drift detectors with shutters integrated deeply into the objective lens), EDX images of the regions on interest were performed with a resolution of 1024x1024 pixels (Figure 4G, H) or 512x512 pixels (Figure 6). These images were generated by the sum of 200 frames with 2.5 µs pixel dwell time at 200 keV (Figure 4G, H) or using 56 frames with 10 µs pixel dwell time at 200 keV (Figure 6).

All images were processed in Adobe Photoshop and line graphs were generated using Excel.

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Lanthanide-conjugated antibodies do not allow EDX-based protein detection in electron microscopy

Chapter 6

Summary, general discussion and
perspectives

Summary and discussion

The human body consists of different types of building blocks, such as proteins, organized at specific localizations within tissues and cells. When proteins get mutated and/or dislocated, diseases may develop. Visualization of these proteins is essential to reveal the protein cellular localization in their natural environment, which contributes to the knowledge about their function. Protein visualization is based on probes that are fluorescence for light microscopy (LM), or electron dense for electron microscopy (EM). Each modality has its strength like large field of views or high resolution, respectively. The two modalities can be combined in the technique correlated LM and EM (CLEM)^{1,2}. Optimal CLEM probes facilitate both fluorescence and electron density for the best image overlay. Examples of CLEM probes are Quantum dots (QDs)³ or FLIPPER (fluorescent indicator and peroxidase for precipitation with EM resolution)⁴ used in affinity-based or genetically encoded labeling, respectively. However, each labeling strategy has certain limitations and a balance is needed to have an efficient labeling and well-preserved ultrastructure. The aim of this thesis is to develop and validate a novel probe for CLEM and improve the ease of targeting in both microscopic modalities.

Affinity-based labeling is used on regular basis to target endogenous proteins. For this conventional immunoglobulin G (IgG) antibody labeling, primary and secondary IgGs, are typically used. This indirect labeling method results in a distance > 25 nm between the protein and tag^{5,6}. To limit this discrepancy, smaller IgG derivatives were pioneered⁷⁻⁹, but they appeared to be less stable and had a decrease affinity¹⁰. Later, nanobodies were used because of their high antigen binding affinity^{5,11}. Nanobodies are derived from heavy-chain only IgGs and are a 10-fold smaller than conventional IgGs^{5,12}. In addition they are easy to handle with molecular cloning tools because, they consists of only a single domain.

Chapter 2 reviews the developments of the nanobody technology in microscopy. Since nanobodies were first implemented in microscopy¹³, different studies showed the advantage of using nanobodies over IgGs, like gaining resolution or targeting live-cell intracellular endogenous proteins. When expressed, nanobodies immediately bind to their target, therefore methods have been developed to control antigen-recognition binding by light activation methods, or by applying purified nanobodies. Nanobodies, however, might negatively influence the intracellular target protein by silencing or delocalize the target, but researchers can use these tools to manipulate the localization of endogenous proteins to have an impact on their function. Overall, this review will inform researchers on the possibilities to use nanobodies in microscopy.

FLIPPER-bodies were developed as a novel nanobody-based CLEM probe in **Chapter 3a**⁶. This probe was generated by the genetic fusion of nanobody- and FLIPPER (FP and peroxidase) encoding cDNAs, after which this new affinity-based probed was produced and secreted by mammalian cells. A proof-of-concept showed the use of FLIPPER-bodies targeting green fluorescent protein (GFP) or growth factor receptor family proteins EGFR or HER2, in both microscopic modalities. When targeting extracellular proteins EGFR and HER2, no permeabilization was applied, resulting in a well-preserved ultrastructure. In addition, the label efficiency with the FLIPPER-body was much higher compared to QDs in a conventional IgG immunolabeling and moreover cell-cell contact site were labeled only when using

the FLIPPER-body, indicating an improved penetration depth. For targeting nuclear GFP localization, the FLIPPER-body shows a fast nuclear labeling, which was not possible with IgGs, but the cells were permeabilized resulting in a damaged ultrastructure. The peroxidase of FLIPPER-bodies added a black precipitation visible in EM, when diaminobenzidine (DAB) was present. The added electron density is more diffused compared to nanoparticles like gold. Therefore, the use of FLIPPER-bodies in targeting rare events in EM will be challenging. Also, the added grey values can have similar values as endogenous electron density present in e.g. lysosomes. Hence, for rare events it might be beneficial to choose nanoparticles fused to nanobodies, but coupling nanoparticles to nanobodies is more laborious and increase the probe size.

Affinity-based probes, like purified FLIPPER-body and IgG, have the disadvantage of a required permeabilization when targeting intracellular proteins. Permeabilization agents, like Triton X-100, dissolve the cellular membranes and although the effect in LM is not seen, the damaged ultrastructure is visible in EM¹⁴. This makes the damaged organelles not visible or hard to distinguish from one and other, resulting in more errors and biased conclusions. Therefore, there is a need to label endogenous intracellular proteins, without compensating the ultrastructure. In **Chapter 3b**, FLIPPER-bodies were used in a labeling approach free of permeabilization, called *NATIVE (nanobody-assisted tissue immunostaining for volumetric EM)*¹⁵. This method is based on using a mild primary fixative pre-labeling, and a heavy fixative post-labeling. In this thesis, different protein fusions with GFP were expressed in cells and mildly fixed using paraformaldehyde. After fixation and FLIPPER-body labeling, the cells were secondary fixed using glutaraldehyde and osmium followed by EM embedding. Specific fluorescence in LM and electron density in EM, as product of the FLIPPER-bodies, was shown and the ultrastructure was better preserved using this method. However, it must be noted that in both modalities a high background was detected. Reducing the background is important to increase the signal-to-noise for better labeling visualization. Especially in EM, the background can be misleading and the results are more prone for misinterpretation. In addition, diffusion of the reaction product may not be excluded¹⁶. Purification of the FLIPPER-bodies might contribute to a better signal-to-noise ratio by better control the applied label concentration. Overall, the NATIVE protocol opens new strategies to label intracellular targets, compromising cellular ultrastructure to a lesser extent than current routine protocols.

Genetically encoded probes do not need harmful permeabilization, and thus the ultrastructure will be preserved. In **Chapter 4**¹⁷, two cytosolic expressed probes were used to study extracellular vesicle (EV) cargo exposure in recipient cells. EVs are small membrane vesicles that can mediate in long-distance cell-cell communication to deliver biomolecules^{18, 19}. Their size is below the diffraction limit of light, which complicates LM studies of single EVs. Here, using cytosolic probes, a discrimination was made between EV induced endosomal lysis and EV fusion with the endosomal membrane. CLEM and IgG staining revealed EV membrane fusion with maturing endosomes. This membrane fusion was inhibited by neutralizing the pH or accumulating cholesterol in the endosomal compartments. Altogether, this leads to a conclusion that once EVs are taken up via endocytosis, EVs undergo a pH- and cholesterol-dependent membrane fusion in maturing endosomes. In this current study, donor and recipient cell both were derived from the same cell type. It is known that EVs differ in membrane composition per cell type²⁰⁻²² and therefore EVs from cell types might

release their cargo differently. However, here general tools have been provided to study such membrane fusion events that are below the diffraction limit of light and can broadly be implemented in other EV research.

The EVs used also contained a peroxidase²³, however, no black precipitation was detected after DAB conversion, suggesting that the peroxidase was not active within the EVs. This complicated EM analysis and CLEM was necessary to distinguish between intracellular endogenous vesicles and the taken up EVs. Still, single EVs in the recipient cells might be missed in the EM analysis because of the diffraction limit of light and the resolution discrepancy between the two microscopic modalities¹. So, it is important in EV localization studies that another EM visible marker is present, for instance gold nanoparticles.

Multi-target labeling in LM is based on using spectral differences of fluorescent proteins (FPs)²⁴ and in EM multi-target labeling is based on different shaped nanoparticle and peroxidases. This limits in EM protein targeting up to three distinguishable signals¹ because use of more QD and gold nanoparticles are hard to distinguish from each other. To enlarge the number of targets per sample, elemental analysis techniques based on X-ray collection were explored to use in life science²⁵. An energy dispersive X-ray spectroscopy (EDX) detector can easily be implemented to a Scanning EM to detect per pixel the elemental composition within the reconstructed EM image. This technique, colorEM, allows to detect EM probes unbiased based on their elemental composition, rather than based on a more biased analysis of their shape and size²⁵⁻²⁷. Addition of rare-earth elements can contribute to multi-targeting EM analysis, which then will be limited by the elements of the periodic table.

In **Chapter 5**, IgGs conjugated to rare-earth heavy-metal polymers were explored as novel probes in EDX²⁸. These IgGs consist of 161 lanthanides. As proof-of-concept two secondary IgGs were fused to lanthanides neodymium (Nd) or terbium (Tb) and with LM analysis it was shown that the antigen recognition of the IgGs were not blocked by the polymers. In addition, EDX analyzation of the pure IgG solutions showed Nd and Tb present. Next, pre- and post- EM embedding labeling experiments were performed and sections of 200 nm were analyzed, but both metals were not detected. Even when analyzing whole cells in SEM prepared sample, the elements could not be detected. This suggests that there are either not enough X-rays produced or collected. To exclude influence of harsh plastic embedding chemicals or elemental wash out, post-embedding labeling according to Tokuyasu²⁹ was done. In addition, to increase the collection of X-rays, a TEM system with three additional detectors was used, nonetheless implementing Tokuyasu and/or adding more detectors did not result in the lanthanide detection. So, to exclude EM preparation side effects in the future, the EM samples need to be validated first using other techniques, like mass cytometry. Finally, even though the EDX detectors have been improved in the last decades it is still possible that they are not sensitive enough to detect these low numbers of elements per IgG. So, increasing the number of lanthanides per IgG will also increase the likelihood on being detected by EDX.

Future perspectives

CLEM protein detection is about finding the balance between labeling efficiency and cellular ultrastructure. Therefore, non-permeabilized labeling methods are preferred. Improving

CLEM data analysis using probes is beneficial to create a perfect overlay between LM and EM. This is better when both fluorescence and electron density are present in the same section. So, combining FLIPPER-bodies with FPs that remains fluorescent after EM plastic embedding would make it possible to image fluorescence and peroxidase on the same EM section.

Current challenges that emerge/remain maybe tackled as follows: mEosEM was recently described as a FP that can withstand osmium staining³⁰, which is the main cause of fluorescence loss during EM embedding. The next challenge in combining both fluorescence and DAB precipitated together in the EM section, is the fluorescent quenching of DAB, however, it is not known yet what the effect of DAB is on mEosEM. Overall, using the mEosEM in FLIPPER-bodies, together with labeling cells free of permeabilization, can contribute in an efficient labeling within both modalities, in ultrastructural preserved cells.

Peroxidases present in the FLIPPER-body convert DAB into electron dense precipitates. A distinction between multiple targets cannot be made using de FLIPPER-body, because they will all lead to a black staining into the grey-scale EM image. In addition, using different peroxidases, like APEX2 and HRP, also are indistinguishable. In order to combine multiple target FLIPPER-bodies, different heavy-metal conjugated DAB²⁷ can be used to allow multi-targeting visualization at EM level, using elemental analysis.

ColorEM experiments based on endogenous elements will have limited information because of the low variance in elements present in cells²⁶. Therefore, adding rare-earth heavy metals will increase the label pallet, which will stand out on the low endogenous background. Nanoparticles like QDs and gold were successfully pioneered^{25, 26}, so to enlarge the colorEM toolbox further, IgGs conjugated to polymers with lanthanides were pioneered, chapter 5. However, with recent conjugation methods there are max 161 elements present per IgG while in a 10 nm gold nanoparticle, there are already >1000 elements present³¹. So, new developments regarding adding more lanthanides per IgG are needed, like addition of more polymers per IgG or more lanthanides per polymer.

Probe development by adding more elements will cause an increase in probe size, which can be a limiting factor in labeling efficiency and resolution. Therefore, even though the EDX detectors have increased in sensitivity in the recent years²⁵, other elemental-guided imaging techniques might be more suitable for ColorEM concerning sensitivity. The advantage of EDX is the easy implementation in combination with the straightforward data analysis. Sensitivity of EDX is however a disadvantage and other elemental-guided imaging techniques are more sensitive, like nanoscale-secondary ion mass spectroscopy (NanoSIMS)^{25, 32, 33} and electron energy loss spectroscopy (EELS)^{25, 27}. In NanoSIMS, the focused ion beam ionizes the sample, followed by collection and identification using mass spectrometry and in EELS, the energy loss of transmitted electrons that interacted with the sample are detected. The limiting factor of both these techniques however, is the complex detection implementation and the laborious data analysis. Hence, it might be of interest to investigate in other elemental-guided imaging techniques for ColorEM. Once ColorEM is successful with heavy-metal conjugated IgGs, a next step in improving the resolution and label efficiency, is coupling the rare-earth heavy-metal polymers to nanobodies. Ultimately targeting all organelles with nanobodies in combination with metals will improve unbiased EM analysis, since it is no

longer based on researcher's interpretation.

Unbiased analysis can be of interest in EV biology. In our current study, chapter 4, analysis was based on morphology and LM IgG analysis. Having an unbiased readout will contribute in not only better analysis, but also unexpected results might be picked up. In this current study, co-localization of the endosomal/lysosomal system was tested, however autophagic route was not included. Autophagic proteins (non-canonical) have been shown to have a function in the biogenesis of EV formation^{34, 35} and EVs where shown to trigger autophagy in recipient cells^{34, 36}. Whether autophagy play a role as well in the EV cargo escape or EV degradation remains to be elucidated.

The cytosolic fluobody used in Chapter 4, is based on an anti-GFP nanobody and is expressed in the cytoplasm. Here, the conditionally stable nanobodies can contribute to degrade unbound fluobodies, resulting in bound-only fluobodies³⁷. This may improve the sensitivity in LM but also for EM. The fluobodies also contained APEX2 for potential EV localization in EM. However, due to the high levels of unbound nanobodies in the cytoplasm, no accumulation of fluobodies were detected at regions of interest, due to too low signal-to-noise ratio. The conditionally stable nanobodies can contribute to better signal-to-noise, to visualize fluobody accumulations at EM level³⁸. This will improve the analysis of membrane structures involved in EV cargo escape and moreover, it further improves the correlation between LM and EM. Finally, to get a better insight into the EV dynamics, the (conditionally stable) fluobody can be combined with live-cell imaging. This will give more information about how fast fusion events happens and if the fluorescence in a later stage remains present or if the fluorescence will be degraded by the lysosomal system. Fixation at the moment of fusion, followed by 3D CLEM, will give more structural information about EV and endosome membrane fusion.

Nanobody-based probes are, despite their advantages, limited used in research. The main limitations are low number of nanobody targets and cDNA/protein accessibility for other researchers³⁹. Development of new nanobody targets need immunization of lama's which is a slow and expensive procedure. A new method was developed to omit lama immunization, but using synthetic made yeast nanobody library⁴⁰. This can decrease the development time of nanobodies, as well as reducing the expenses. Sharing the nanobodies cDNA will contribute to implementation by other researchers, contributing to the applicability and the reproducibility, as was seen with GFP⁴¹. Thus, newly developed small protein probes for CLEM, can become universal and easy adjustable probes to the researchers wishes, and thereby contribute to the understanding of building blocks of life in the human body.

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Appendix

Nederlandse samenvatting

Dankwoord

About the author

Nederlandse samenvatting

Het menselijk lichaam bestaat uit verschillende bouwstenen, zoals eiwitten, die specifiek zijn georganiseerd binnen verschillende weefsels en cellen. Wanneer eiwitten muteren en/of van lokalisatie veranderd zijn kunnen er zich ziektes ontwikkelen. Het zichtbaar maken van deze eiwitten is belangrijk om zo de lokalisatie te achterhalen in hun natuurlijke omgeving, wat een bijdrage levert aan de ontdekking van de eiwitfunctie. In microscopie worden eiwitten zichtbaar gemaakt door ze te voorzien van labels, ofwel probes, die vaak fluorescent zijn in lichtmicroscopie (LM) en/of een hoge elektronendichtheid hebben voor elektronenmicroscopie (EM). Elk van deze technieken heeft zijn eigen voordelen, zoals een groot gezichtsveld of hoge resolutie. Deze sterke eigenschappen kunnen worden gecombineerd in de techniek genaamd gecorreleerde licht- en elektronenmicroscopie (CLEM). De ideale probe voor CLEM zorgt dan zowel voor fluorescentie als voor hoge elektronendichtheid. Voorbeelden van zulke probes zijn Quantum dots (QDs) of FLIPPER (*fluorescent indicator and peroxidase for precipitation with EM resolution*), die worden gebruikt in affiniteit- of genetisch gebaseerde labeling van eiwitten. Echter elk van deze labeling strategie zijn eigen beperkingen en zal er gezocht moeten worden naar de balans tussen een efficiënte labeling en het behouden van een goede ultrastructuur. Het doel van dit proefschrift is om nieuwe probes te ontwikkelen en toe te passen voor CLEM en daarbij het eiwit te labelen in beide microscopie technieken te verbeteren.

Affiniteit-gebaseerde probes kunnen worden gebruikt om endogene eiwitten aan te tonen. Vaak worden hiervoor conventionele immunoglobuline type G (IgG) antilichamen gebruikt, door middel van een primair IgG in combinatie met een gelabelde secundair IgG. Deze indirecte labeling-methode leidt tot een afstand van >25 nm tussen het eiwit en het label. Om deze afstand te beperken worden er kleine IgG-afgeleiden gebruikt, maar deze bleken minder stabiel en de affiniteit was afgenomen. Later zijn nanobodies geïntroduceerd vanwege hun hoge stabiliteit en affiniteit. Dit zijn antigeen-bindende eiwitten die afgeleid zijn van “zwarte-keten” IgG's, afkomstig van kameelachtigen. Nanobodies zijn 10 keer zo klein zijn als conventionele IgG's. Daarnaast zijn nanobodies gemakkelijk te gebruiken in moleculaire kloner strategieën, doordat ze bestaan uit één enkel domein.

Hoofdstuk 2 biedt een overzicht van de ontwikkelingen van de nanobody-technologie binnen microscopie. Sinds de introductie van nanobodies in microscopie laten verschillende studies hiervan de voordelen zien boven het gebruik van IgG's, zoals het toenemen van de resolutie en het visualiseren van levende endogene eiwitten in de cel. Tijdens de translatie van nanobodies kunnen ze direct binden met hun doel/antigeen. Er zijn daarom methodes ontwikkeld om de eiwitexpressie gecontroleerd te laten activeren of door gebruik te maken van gepurificeerde nanobodies. De binding van nanobodies met het eiwit kan ook een negatieve invloed hebben op het eiwit door het te deactiveren of door de lokalisatie te veranderen. Echter, onderzoekers kunnen dit ook juist gebruiken om specifieke eiwitten te manipuleren om het effect op de gehele cel te kunnen analyseren. Dit overzicht laat onderzoekers zien wat de mogelijkheden zijn voor het gebruik van nanobodies en microscopie.

FLIPPER-bodies zijn ontwikkeld als nieuwe nanobody gebaseerde CLEM probes in **hoofdstuk 3a**. Deze probes worden gecreëerd door de genetische fusie van nanobody-

en FLIPPER (fluorescent eiwit en peroxidase) gecodeerd cDNA, waarna deze nieuwe affiniteit-gebaseerde probe geproduceerd en uitgescheiden wordt door zoogdiercellen. Een conceptstudie laat zien dat FLIPPER-bodies succesvol zijn in het visualiseren van groen fluorescent eiwit (GFP) of in de groeifactor receptor eiwitfamilie (EGFR en HER2) in beide microscopie technieken. Extracellulaire eiwitten EGFR en HER2 hebben als antigeen geen permeabilisatie nodig waardoor de ultrastructuur bewaard blijft. Ook laat deze studie zien dat de label efficiëntie met de FLIPPER-body hoger is in vergelijking met QDs in een conventionele IgG immunolabeling en bovendien zijn de cel-cel contacten alleen gelabeld wanneer FLIPPER-bodies gebruikt worden, wat een verbeterde penetratie-diepte laat zien. Wanneer nucleair gelokaliseerd GFP wordt gelabeld, zien we een snelle kleuring met FLIPPER-bodies, wat niet mogelijk is met de IgG's. De cellen moeten hiervoor wel gepermeabiliseerd worden, wat een nadelig effect heeft op de ultrastructuur. De peroxidase van de FLIPPER-body voegt samen met diaminobenzidine (DAB) een zwarte neerslag toe die zichtbaar is in EM. Deze toegevoegde elektronendichtheid is meer diffuus vergeleken met nanodeeltjes zoals nanogoud. Dit maakt het detecteren in EM van weinig voorkomende eiwitten moeilijker. Ook kunnen de toegevoegde grijswaarden in hetzelfde bereik vallen als grijswaarden van endogene structuren zoals lysosomen. Hiervoor zou het voordelig kunnen zijn om nanodeeltjes te fuseren met nanobodies, alleen is dit arbeidsintensiever en de label grootte neemt toe.

Affiniteit-gebaseerde probes, zoals FLIPPER-body en IgG, hebben het nadeel dat permeabilisatie nodig is in het geval van intracellulaire antigenen. Permeabilisatie reagens lossen cellulaire membranen op, wat niet zichtbaar is met LM, maar met EM is de beschadigde ultrastructuur wel te zien. Dit maakt het moeilijk om verschillende organellen te kunnen onderscheiden, wat resulteert in meer fouten in de data-analyse. Daarom is het nodig om endogene, intracellulaire eiwitten te kunnen labelen zonder de ultrastructuur te beschadigen. In **hoofdstuk 3b** zijn FLIPPER-bodies gebruikt in een permeabilisatie-vrije methode genaamd *NATIVE (nanobody-assisted tissue immunostaining for volumetric EM)*. Deze methode is gebaseerd op het gebruik van een milde primaire fixatie voor de labeling en een zwaardere fixatie na de labeling. In dit proefschrift wordt gebruik gemaakt van verschillende eiwit- en GFP-fusies die gefixeerd worden met paraformaldehyde. Na deze fixatie en FLIPPER-body labeling worden de cellen gefixeerd met glutaraaldehyde en osmium, gevolgd door een EM inbedding. De FLIPPER-body laat een specifieke fluorescentie in LM en elektronendichtheid in EM zien en ook de ultrastructuur is beter bewaard gebleven. Echter is er wel in beide microscoop modaliteiten een hoge achtergrondkleuring te zien. Het is belangrijk om dit te verlagen om een betere signaalruisverhouding te krijgen en daarmee een betere eiwit visualisatie. Dit is vooral van belang in EM, waar de achtergrond misleidend kan zijn en kan leiden tot misinterpretatie. Bovendien kan bij een hoog achtergrondsignaal diffusie van DAB niet worden uitgesloten. Het opzuiveren van de FLIPPER-body kan bijdrage aan een betere signaalruisverhouding doordat de concentratie van de probe beter te controleren is. De NATIVE-methode maakt nieuwe strategieën mogelijk om intracellulaire antigenen te labelen met als voordeel dat de ultrastructuur van de cel minder is aangetast in vergelijking met huidige methoden.

Genetisch gecodeerde probes hebben geen permeabilisatie nodig om aanwezig te zijn in de cel, waardoor de ultrastructuur van de cel in tact blijft. In **hoofdstuk 4** worden twee probes gebruikt die tot expressie komen in het cytosol om te onderzoeken hoe extracellulaire

membraanblaasjes (vesikels; EV) hun inhoud delen met ontvanger cellen. EV's zijn kleine membraanblaasjes die onder andere lange afstand cel-cel communicatie mogelijk maken door het afgeven van biomoleculen. Hun grootte is beneden de diffractielimiet van licht, wat het bestuderen met LM moeilijker maakt. In dit hoofdstuk is door middel van cytosolische probes het onderscheid gemaakt tussen EV geïnduceerde endosoom lysis en EV fusie met het endosoom-membraan. CLEM en IgG kleuring laten EV membraanfusie zien met de endosoom. Deze membraanfusie wordt geblokkeerd door het neutraliseren van de pH of het verhogen van cholesterol in de endosoom compartimenten. Dit leidt tot de conclusie dat, wanneer een EV is opgenomen door de cel via endocytose, de EV een pH- en cholesterol- afhankelijke membraanfusie ondervindt met het endosoom-membraan. In het huidige onderzoek zijn de donor en ontvangende cel van hetzelfde type. Het is bekend dat de EV membraan compositie per cel type anders is en daarom kunnen EV's van andere cel types hun inhoud anders vrijgeven. Echter er wordt hier een algemene methode beschreven om dit soort membraanfusie te laten zien en deze kan geïmplementeerd worden in andere EV-studies.

De EV's, gebruikt in hoofdstuk 4, bevatten ook peroxidases, maar na het toevoegen van DAB is er geen zwarte neerslag zichtbaar wat suggereert dat de peroxidase in deze EV's niet actief zijn. Dit maakt de EM-analyse moeilijker, zodat CLEM nodig is om het verschil zichtbaar te maken tussen endogene vesikels en opgenomen EV's. Hierdoor is het mogelijk dat niet alle EV's zijn geanalyseerd door het resolutie verschil van de twee microscopie technieken. Zodoende is het belangrijk om EV lokalisatie studies te doen met andere labels die zichtbaar zijn in EM zoals goud nanodeeltjes.

Waar het labelen van meerdere antigenen in LM is gebaseerd op het gebruik van spectraal verschillende fluorescente eiwitten (FP's), is dit in EM gebaseerd op het toepassen van verschillend gevormde nanodeeltjes en peroxidase. In EM limiteert dit eiwitlabeling tot drie verschillende antigenen omdat de nanodeeltjes QDs en goud moeilijk uit elkaar te halen zijn¹. Om dit labelaantal te vergroten zijn element analysetechnieken gebaseerd op X-rays voor het gebruik in biowetenschappen onderzocht. Een electron dispersive X-ray spectroscopie (EDX) detector kan gemakkelijk worden toegevoegd aan een scanning EM (SEM) om per pixel de element compositie te reconstrueren bovenop een EM afbeelding. Deze techniek "kleurenEM", maakt het mogelijk om EM probes te detecteren op basis van de element compositie in plaats van een analyse op basis van hun morfologie. Het toevoegen van zeldzame elementen kan bijdragen aan het zichtbaar maken van meerdere eiwitten met EM-analyse, wat dan gelimiteerd is aan de elementen in het periodieke systeem.

In **hoofdstuk 5** zijn IgG's gelinkt met polymeren die zeldzame aardmetalen bevatten om te onderzoeken of deze probes geschikt zijn voor EDX. Hierbij bevat een enkele IgG maximaal 70 lanthanide. In een conceptstudie zijn twee secundaire IgG's gelinkt met lanthanide neodymium (Nd) of terbium (Tb) en is met LM aangetoond dat de antigeen herkenning van de IgG niet is geblokkeerd door de polymeren. Daarnaast laat een EDX-analyse van het pure IgG zien dat Nd of Tb aanwezig is. Vervolgens zijn pre-EM inbedding labeling experimenten gedaan en zijn coupes van 200 nm geanalyseerd. Echter kon er geen lanthanide worden gedetecteerd met EDX-analyse. Zelfs wanneer er hele cellen worden gebruikt die voor SEM klaar gemaakt zijn, kunnen de elementen niet worden gedetecteerd. Hierdoor lijkt het dat er niet genoeg X-rays worden geproduceerd of gedetecteerd. De invloed van chemicaliën

of uitwassen van de elementen tijdens EM inbedding is uitgesloten door een post-EM inbedding labeling uit te voeren volgens Tokuyasu. Daarnaast is er een EM gebruikt waarbij meerdere detectoren zijn gebruikt om het aantal gedetecteerde X-rays te verhogen. Echter resulteerden beide testen niet in de detectie van de lanthaniden. Om het effect van EM inbedding volledig te kunnen uitsluiten zal hetzelfde sample eerst met mass cytometry gevalideerd moeten worden. Tot slot, ook al zijn EDX-detectoren in de afgelopen jaren al verbeterd, blijft het mogelijk dat in de komende jaren de sensitiviteit van de detectoren verbeterd zal worden.

Samenvattend laat dit proefschrift de diversiteit van eiwitlabeling met nanobodies zien voor verschillende microscopie technieken. Een nieuwe probe – FLIPPER-body – laat een efficiënte kleuring zien in meerdere microscopie technieken, waarbij de voordelen ten opzichte van conventionele IgG's duidelijk worden. Deze kleine probe voor CLEM kan universeel worden gebruikt, doordat het gemakkelijk aan te passen is aan de individuele wensen van onderzoekers. Met deze nieuwe labeling methode is het gebleken hoe EV's hun inhoud kunnen vrijgeven, waarmee bewezen is dat deze nieuwe probe een bijdrage kan leveren aan het begrijpen van de bouwstenen in het menselijk lichaam.

Dankwoord

Veel trots, en toch ook een gevoel van opluchting. Blij dat het mij is gelukt om deze thesis met succes af te ronden. Dit ging gepaard met hulp vanuit vele kanten, en daarom wil ik in het bijzonder een aantal mensen bedanken.

Allereerst wil ik de “*kiepmanslab*” bedanken voor een mooie tijd in het hoge noorden.

Jeroen, jij had allang in de gaten op de manier waarop ik je naam zei dat ik een “vraagje” had voor je. Ik was dan ook erg blij dat jij mijn buurman was, en wil ik je bedanken voor al die antwoorden die ik nodig had.

Anouk, mede door jou als collega, maar vooral als vriendin, heb ik echt genoten van mijn tijd in Groningen. Bedankt voor alle gezelligheid op werk, en alle gezellige momenten daarbuiten, vooral de borrels in het pakhuis blijven me bij.

Nicole, bedankt voor jou (typisch Amerikaanse?) enthousiasme die een positieve uitwerking had op mij, zowel op werk als daarbuiten. Heel veel succes met de laatste loodjes voor jou thesis. Ik weet dat je het kunt!

Pascal, bedankt voor alle feedback en input die nodig zijn geweest om deze thesis succesvol af te ronden. Zal je ooit die Elfstedentocht gaan fietsen?

Klaas, bedankt voor alle trainingen op de licht microscopen en voor al die keren dat ik je hulp daarbij nodig had.

Kim, Arianna en Peter wil ik bedanken voor de gezellige tijd in de groep.

Jouke en Maaike, bedankt voor jullie bijdrage aan deze thesis tijdens jullie stage.

En tot slot **Ben**, allereerst bedankt voor het vertrouwen in mij en in het begeleiden van mijn academische ontwikkelingen. Jouw kritische blik op mijn werk lieten mij nadenken en de kijk op onderwerpen veranderen, jouw enthousiasme op mijn werk maakte ook mij enthousiast, en jouw sturing zorgde voor een succesvolle afloop. Op zowel werk als persoonlijk vlak heb ik veel van je geleerd waar ik je erg dankbaar voor ben.

Sven, bedankt voor jou adviezen, discussies en suggesties om deze thesis te verbeteren.

Reggiori lab, I’m really glad you all came to Groningen for science and thanks to all of you for the joyful events we went to.

Bhagysahree and Inge, thank you both for the fruitfull collaboration. Bhagyashree, I really appreciate your mentality, determination and work ethics.

Mijn lieve **familie**, Paps & Mams, Sanne, Michiel wil ik vooral bedanken voor de mentale support: het vertrouwen en het meeleven in periodes van stress. Toch wil ik in het bijzonder mijn broer **Michiel** bedanken, die inmiddels vele hoofdstukken woord voor woord erg goed kent.

Ook erg belangrijk voor mijn mentale support zijn mijn vrienden geweest. Lisanne, Loes, Marjolein, Karina & Deniz, *“the day after”* bedankt voor jullie steun op de nodige momenten en de momenten waarbij we juist even niet over werk praatte, maar gewoon even gelachen hebben. De keren dat mijn stress niveau werd meegenomen in waar en wanneer ons weekendje weg zou plaats vinden, de kleine gebaren waardeer ik enorm. Nathalie en Kimberley, *“van Heesch zusjes”* bedankt voor jullie support en voor alle gezellige spelletjes avonden en voor het bewijs dat afstand niet uitmaakt binnen een vriendschap. *Dames 18*, bedankt voor al die sportieve, gezellige en onvergetelijke momenten samen.

Allemaal bedankt!

Marit

About the author

Marit de Beer was born on the 4th of January, 1991, in Tilburg, the Netherlands. After graduating from secondary school in 2008 (HAVO, at Beatrix college, Tilburg), she studied for a Bachelor life science (2008-2012, at Hogeschool van Arnhem en Nijmegen, Nijmegen). Here, she did an internship at the department of cell biology in the UMC Utrecht, in the group of Prof. Judith Klumperman, supervised by Viola Oorschot, where she learned the skills for electron microscopy. Subsequently, Marit went to the Radboud university to follow a (pre-) master Medical biology (2012-2015), which included two research projects. The first project was in the group of Prof. Peter Friedl under the supervision of Dr. Jack Fransen and Dr. Bettina Weigelin at the department of cell biology of the Radboudumc, where she worked on a project titled *“Tissue microstructures guiding collective tumor migration”*. For the second project, at the department of cell biology of the University Medical Center Groningen (UMCG), Marit joined the group of Dr. Ben Giepmans, where she worked on a project titled *“Engineering new small protein probes for correlative microscopy”*.

In September 2015, Marit started her Ph.D. project within the department of cell biology (currently biomedical sciences of cells & systems) at the UMCG in the group of Dr. Ben Giepmans. Her Ph.D. project was part of a larger project “Microscopy Valley”, funded by NWO Stichting Techniek en Wetenschap (STW). Within her project, Marit continued on her work performed during her Master project: the development of probes for correlated microscopy (CLEM), based on small protein probes, as described in this thesis. Following her Ph.D. project, Marit started in November 2019 a postdoctoral fellowship at the Radboudumc in Nijmegen, in the group of Prof. Dr. Nico Sommerdijk. This project is focused on the development of 3D cryo-CLEM, and will be implemented in mineralization of bone.