Leibniz-Gemeinschaft

Final report

Electron microscopy of labeled protein complex subunits in whole cells in aqueous environment

Leibniz-Institute: INM - Leibniz Institute for New Materials

Reference number: SAW-2014-INM-1

Project period: 3.2014 - 2.2017

Contact partner: Prof. Dr. Niels de Jonge

Stand: 04. Dezember 2015

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Summary

A central challenge of the life sciences is to understand the organization of protein complexes driving cellular function. This knowledge is needed, for example, to effectively fight diseases, such as cancer, and virus infections. Understanding how protein complexes are organized and interact at the nanoscale within the crowded and compartmentalized volume of the cell is presently limited by the available microscopy techniques. Within this project funded by the Leibniz association, our team including Dr. Diana B. Peckys pioneered a novel concept to study the stoichiometry and distribution of membrane proteins within intact mammalian in their native liquid environment. Membrane proteins were labeled with nanoparticles, and the cells were imaged with liquid-phase scanning transmission electron microscopy (STEM).

The main aim of the project was to develop the technology from its stage of proven concept into a mature microscopy platform for the study of membrane proteins in mammalian cells. This goal was achieved by i) gaining in-depth understanding of the physics of image formation, ii) determining the resolution on cellular samples for an electron dose within the limit of radiation damage, and iii) demonstrating that the technology is capable of addressing important biological questions.

The liquid-phase STEM technology overcomes key limitations of other analytical methods in the study of cellular function at the molecular level. Nanoscale resolution (2-3 nm) was achieved well within the limit of radiation damage. Crucial for the study of cell function is the capability to screen hundreds of cells and to investigate selected tens of cells with high spatial resolution. This was achieved by combining fluorescence microscopy with liquid-phase STEM and correlating the obtained information. Liquid-phase STEM was used to examine three bio-medically relevant membrane proteins, TMEM16A, ORAI1, and HER2, while they remained in the intact plasma membrane. This research was conducted together with our scientific partners, Prof. Veit Flockerzi, Prof. Stephan Hofmann, Dr. Ulrike Korf, Prof. Stefan Wiemann, and Prof. Barbara A. Niemeyer. An important outcome in cancer research was obtained showing that the HER2 receptor exhibits a different behavior in small subpopulations of breast cancer cells. Liquid-phase STEM is a new analytical method providing unique information for cell biology, and biomedical research, with particular relevance for cancer research because it is capable of addressing receptor functioning at the single cell *and* single molecule level while addressing cancer cell heterogeneity.

Final goal and objectives of the project

The main aim (final goal) of the project was to develop the groundbreaking liquid-phase STEM technology from its past stage of proven concept into a mature microscopy platform for the study of protein complexes and their distribution in eukaryotic cells. To achieve this goal it was deemed necessary to understand the physics of image formation, and to precisely determine the resolution on cellular samples for an electron dose within the limit of radiation damage. Subsequently, it was planned to address an important biological question about the stoichiometric structure of the ion channel formed by the transient receptor potential channel, subfamily V, member 6 (TRPV6) protein, with relevance for oncology, in collaboration with a scientific partner working in the life sciences, Prof. Dr. Veit Flockerzi, Saarland University. The research was of an interdisciplinary nature, and comprised following three objectives

1. to develop a quantitative understanding of the physics of image formation of liquid-phase STEM,
2. to determine the resolution of liquid-phase STEM of cells, and
3. to study TRPV6 oligomerization.

Development of the project

The following describes the work executed in each objective (O).

Research in O1

Test samples were prepared with the goal to measure the spatial resolution as function of the vertical location within a sample, and other experimental parameters. But instead of using liquid-based samples, we found it easier to use solid aluminum samples with gold nanoparticles embedded in layers at different vertical positions.[3, 12, 191] Since aluminum has a rather low atomic number, it was possible to compare the results with those obtained in liquid.

STEM measurements were conducted as planned but for a different sample than originally planned for practical reasons.[3, 12, 19] The planned environmental scanning electron microscopy (ESEM) STEM measurements were carried out using cellular samples with embedded gold nanoparticles.[11, 17]

Extensive theoretical work conducted to develop a full model of the spatial resolution of liquid-phase STEM as function of the involved parameters, in particular, sample thickness, vertical location of nanoparticle in the liquid, and electron dose. Instead of a full analytical model, it was found more precise to solve starting equations in a numerical manner. The theoretical framework is now ready and is capable of calculating the spatial resolution for any relevant sample configuration, and microscope settings.[1] The model fitted well with the experimental data.[3] Monte Carlo simulations were not necessary.

Conclusion: The objective was achieved in full.

[1] Number refers to publication list below, numbering reflects order of publication year.

Research in O2

Epidermal growth factor receptors (EGFRs) were considered as suitable membrane protein for testing. Several different strategies were optimized or newly developed for specific labeling of the protein with a nanoparticle for detection with STEM. The experiments were carried out for COS7 fibroblast cells. Quantum dot (QD) nanoparticles were attached in a two-step labeling protocol avoiding label-induced clustering.[16] Gold nanoparticles of 10-nm diameter were attached via a different protocol.[11, 17] We also tested smaller gold nanoparticles but these experiments were not successful until now, and this work has been saved for later. In addition to the original plan, we also developed a label for a different membrane protein, HER2, a family member of EGFR with relevance for cancer research, and these experiments were carried out for SKBR3 breast cancer cells,[15] see Figure 1.

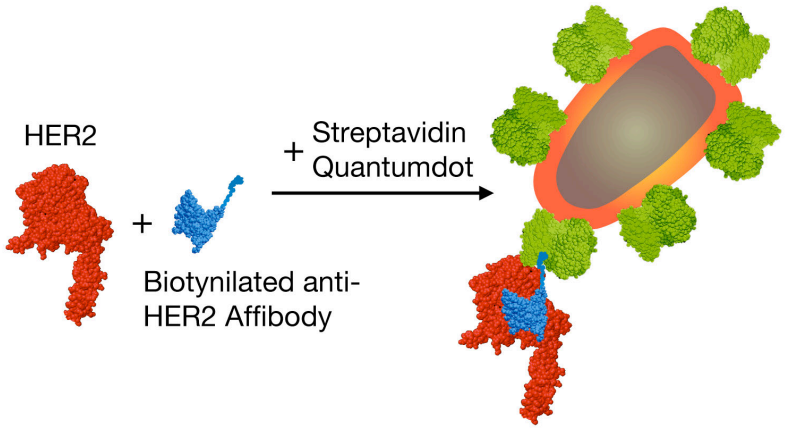

Figure 1. *Model of the biotinylated anti-HER2 affibody (blue) binding to a single epitope of HER2 (red). The single biotin moiety of the affibody binds a streptavidin (green) conjugated to a bullet-shaped quantum dot. From publication Science Advances 1:e1500165, 2015.* [15]

Extensive research was carried out to explore the electron dose limit of radiation damage for ESEM STEM at 30 keV beam energy.[11, 15, 17] This work included developing a method to measure the influence of radiation damage. It was found that the imaging of gold nanoparticles or QDs on fixed COS7 cells in liquid is safely accomplished up to an electron dose of 10^3 $e^-Å^2$, which is considered a large value for the imaging of biological material. However, the minimum dose needed to see the labels was ~3 $e^-Å^2$, and because this dose is still much larger than the critical dose for living cells, it was concluded that live cell imaging was impossible, and those planned experiments were consequently abandoned. To test for possible additional artifacts, we also tested the behavior of the gold nanoparticles in liquid under electron beam irradiation.[19]

The achievable resolution for detecting nanoparticle labels in COS7-, and SKBR3 cells was extensively tested.[11,15, 17] It was found that a spatial resolution of 3 nm is possible well within the limit of radiation damage. This resolution was sufficiently large, so that it was possible to determine the stoichiometry of the membrane protein complexes. [15, 16, 18]

In order to study labeled cells with dedicated STEM at 200 keV beam energy, we run into the complication that the liquid flow holder in our lab did not function well with the cells. After loading the specimen, the sample was too thick and it was impossible to obtain a good resolution with STEM. Therefore, we have developed an alternative liquid enclosure based on a graphene sheet covered the cells using special graphene provided by the group Prof. Hofmann (see below).[2, 7, 13] Here, we tested QD labeling of HER2 in SKBR3 cells. The resolution was studied as function of the electron dose, and was found to be excellent (< 2 nm) even for micrometers-thick cellular regions.

Conclusion: The objective was achieved mostly but live cell STEM was not feasible.

Research in O3

We transfected COS7 cells with TRPV6 cDNA containing the fluorescent protein mCherry at the C-terminal, and a streptavidin-binding peptide (SBP)-Tag sequence in the first extracellular loop. TRPV6 proteins oligomerize into calcium-selective cation channels in the plasma membrane. The DNA was provided by the group of Prof. Flockerzi. The cells were labeled and imaged with ESEM-STEM. However, we found only very few labels, and it was concluded that the protein was not sufficiently abundant.

The project was then changed into studying a different channel, TMEM16A, a membrane protein forming a calcium-activated chloride channel. Three different samples were examined, two with TMEM16A exhibiting a SBP tag for label binding but at a slightly different position, and one in which TMEM16A with SBP tag was mixed with TMEM16A without tag.[4] These samples were imaged with ESEM-STEM. Many tens of cells were imaged, and the locations of 19,583 individual proteins were automatically detected in hundreds of images using software of local design.

A statistical analysis was performed based on the pair correlation function, which measures the probably of particle distances. Also, the amounts of labels in groups of specific size were analyzed. These results were compared with mathematical models predicting the densities of groups of specific sizes as function of the label efficiency, and for a certain hypothesis about the protein stoichiometry. It was eventually concluded that hTMEM16A resides in the plasma membrane as dimer only and is not present as monomer.[4]

In addition to the planned research, we have also developed a label for the calcium channel ORAI1, and successfully analyzed the stoichiometry of this channel,[10] together with the group of Prof. Niemeyer.

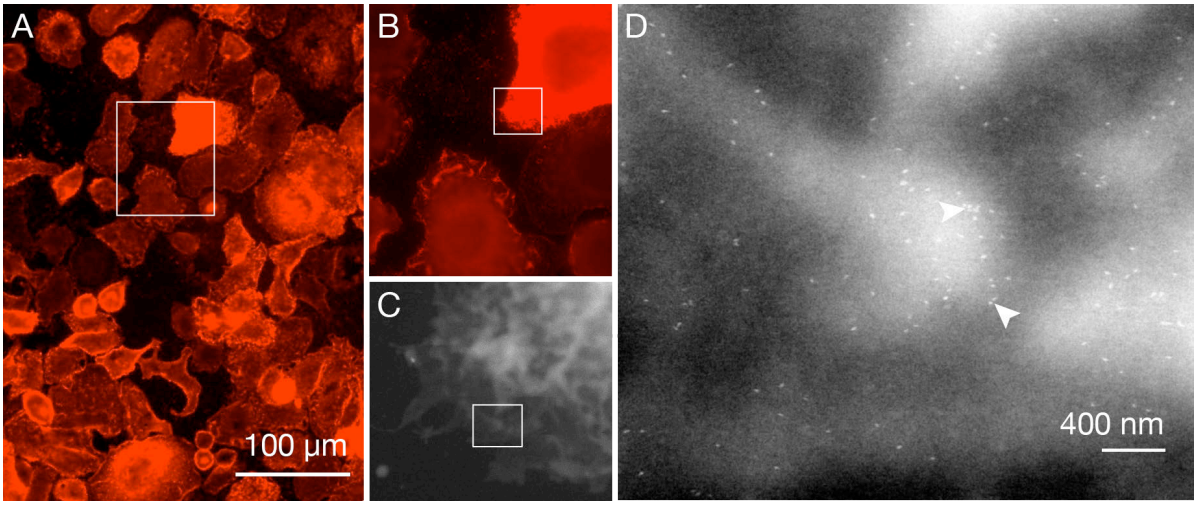

Figure 2. *Correlative light and electron microscopy overview images of QD labeled HER2 on SKBR3 human breast cancer cells. (A) Fluorescence overview image showing several dozens of cells. Individual cells exhibit a high degree of heterogeneity in their morphology and HER2 membrane expression. (B) Fluorescence image of the cells within the boxed area in A. (C) Liquid-phase STEM image of the boxed region in B recorded at 15,000 × magnification using ESEM. (D) STEM image recorded in the boxed region shown in C at 75,000 × magnification. The locations of individual HER2 receptors labeled with QDs are visible as the bright spots. Many pairs (homodimers) are visible; two are indicated with the arrowheads. The brighter background features represent membrane ruffles. Note that the image looks much different than conventional electron microscopy images showing the cellular ultrastructure. From publication Science Advances 1:e1500165, 2015.*[15]

In addition to the planned research, liquid-phase STEM was applied to study the stoichiometry and local variations in the stoichiometry of HER2 in SKBR3 breast cancer cells,[5, 14, 15] see Figure 2. These results revealed a small subpopulation cancer cells exhibiting a different stoichiometry of HER2 than for the bulk, and those cells responded differently to the prescription drug trastuzumab. Possibly, this finding points toward a clue towards addressing the urgent problem of drug resistance development.

Conclusion: The objective to study the stoichiometry of TRPV6 was not achieved but instead a different calcium-activated chloride channel was investigated. In addition, a calcium channel was examined, and liquid-phase STEM was applied to cancer research.

Relevance of the achieved results and future prospects

New analytical method to study membrane proteins

Within the project funded by the Leibniz association, our team pioneered a novel concept to study the stoichiometry and distribution of membrane proteins within intact mammalian in their native liquid environment. The liquid-phase STEM technology overcomes key limitations in the study of cellular function at the molecular level. Nanoscale resolution is achieved well within the limit of radiation damage. Crucial for the study of cell function is the capability to screen hundreds of cells and to investigate selected tens of cells with high spatial resolution in the range of 3 nm. This was achieved by combining fluorescence microscopy with liquid-phase STEM and correlating the obtained information.

We have now a full theoretical understanding of the spatial resolution as function of the relevant experimental parameters. The radiation limit for imaging cells has been determined, and a spatial resolution in the range of 2-3 nm has been demonstrated well within the limit of radiation damage. Liquid-phase STEM has been used in O3 to examine three highly relevant membrane proteins, TMEM16A, ORAI1, and HER2, while they remained in the intact plasma membrane. An important outcome in cancer research was obtained demonstrating both the power of liquid-phase STEM and the need to address cancer cell heterogeneity when examining protein function. Liquid-phase STEM is now in a mature state, and it can be used by a broad range of biological scientists.

It should be emphasized that membrane proteins should be examined at the single molecule and single cell level, while they remain within the plasma membrane and while examining many tens of cells such that cell heterogeneity is taken into account, as is now possible with liquid-phase STEM. It can be expected that the technology will find its place amongst state-of-the-art analytical method for cell biology and biomedical research, thereby providing unique information.

Why is this important? Our knowledge of the functioning of membrane proteins is limited and many open questions exist, for example, about the early stage of signal transduction of EGFR and other receptors, and about the functioning of the HER2 receptor and in particular considering variations between cancer cells. This lack of knowledge is rooted in the limitations of the used analytical methods (Curr. Opin. Struct. Biol. 29, 95 (2014); Cold Spring Harb. Perspec. Biol. 6, a020735-1-13 (2014); Biochim. Biophys. Acta. 1848, 1359 (2015).

A key challenge to understanding the function of membrane proteins is that it is notoriously difficult to study them. The information about the stoichiometry of protein complexes is generally not obtained from intact cells but via biochemical methods involving extraction of proteins from cellular material, via X- ray crystallography of protein crystals or other technologies. First, protein material is pooled from many thousands of cells and thus most knowledge about cellular function is based on population averages, insufficient for studies including cell heterogeneity, which can manifest in large differences in the composition of membrane re-

ceptor expressions between cells of, for example, the same tumor. Second, extracting the membrane proteins from the plasma membrane may lead to artefacts in conclusions about function, because the actual molecular behavior of the receptors is not studied in a native environment. Protein extraction may lead to changes in folding, protein complexes that are bound together in the plasma membrane may not necessarily remain together when extracted, and finally, certain proteins may be difficult to extract. A range of microscopy techniques exist for the study of cells but ***none of these provides localized stoichiometric information for endogenously expressed proteins***. Table 1 lists the most important analytical techniques including their limitations preventing to study HER dimerization in cellular subpopulations.

Table 1. *Important analytical techniques used to study the functional state of proteins (stoichiometry) and their limitations, see also the supplementary discussion of Science Advances 1:e1500165, 2015.*[15]

Technique	Limitation
Biochemical methods	Limited to pooled cellular material, proteins do not remain in cells
	Provides information about average responses in a cell population only
Light microscopy	Spatial resolution insufficient to directly view stoichiometry
	Indirect techniques such as FRET lead to artifacts, for example, detection of back-to-back neighbors rather than subunits in protein complexes
Flow cytometry	Cells not in adherent state
	Prone to artifacts when determining protein stoichiometry
Electron microscopy	Samples in vacuum, thus cells not intact
	Thin cell- or tissue sections needed, challenging to image intact plasma membrane, provides information about few (sections of) cells only
Proximity assay	Does not detect dimers but reflects overall protein proximity, which is heavily influenced by protein concentrations, leads to artifacts

Follow-up funding

This projects has formed the basis to develop liquid-phase STEM for studying membrane proteins within intact mammalian cells. The obtained results and publications formed the basis for two new projects that are now funded.

1. de Jonge, N., Investigation of the influence of breast cancer drugs on HER2 dimerization at the molecular level in individual cells aiming to find clues for causes of drug resistance. funded by the Else Kröner-Fresenius-Stiftung, 2018-2020.
2. de Jonge N., Niemeyer, B.A.. Determination of ORAI channel subunit stoichiometry by visualizing single molecules using STEM. Project of theSFB 1027, Cell Physics, 2017-2020.

Prospects of future commercialization

Our results obtained with the liquid-phase STEM technology clearly demonstrate the need to supplement biomedical research, based on biochemical methods and high throughput screening methods examining the average response of cells, with the new type of 'high content screening' provided by liquid-phase STEM in order to examine the 'exceptions to the rule' in cell response. We expect to trigger the awareness that not only genomic features but also proteome-related ones, such as receptor responses, can and should be examined at the single molecule level in single cells, thereby taking cell heterogeneity into account, which is currently challenging on account of lacking analytical methods. Such analytical approach is particularly relevant for cancer research. Our groundbreaking approach is applicable for a broad range of cell biological and biomedical research involving studies of membrane proteins. Commercialization is required to bring the technology to large-scale scientific usage, and possibly find entry in clinical testing. Prof. de Jonge and Dr. Peckys have developed several strategies for future commercialization of the liquid-phase STEM method, for which 2 patent applications were submitted by the INM in 2014.

Patents

1. de Jonge, N. & Peckys, D.B., Vorrichtung und Verfahren für die stöchiometrische Analyse von Proben, German Patent Application, WO2015/188814 A1 (2015).
2. de Jonge, N. & Peckys, D.B., Spezifische Proteinmarkierung sowie Verfahren zur Identifizierung der statistischen Verteilung der Proteinstöchiometrie, German Patent Application, WO2015/197050 A1 (2015).

Scientific collaborations

The project was conducted in collaboration with the following groups:

1. Group of Prof. Dr. Veit Flockerzi, Department of Experimental and Clinical Pharmacology and Toxicology, Saarland University, 66421 Homburg
2. Group of Prof. Dr. Stephan Hofmann, Engineering Department, University of Cambridge, Cambridge CB3 0FA, United Kingdom
3. Groups of Dr. Ulrike Korf, and Prof. Dr. Stefan Wiemann, Division of Molecular Genome Analysis, German Cancer Research Center, Heidelberg, Germany.
4. Group of Prof. Dr. Barbara A. Niemeyer, Molecular Biophysics, Saarland University, CIPMM, 66421 Homburg, Germany

Qualifying works

N.a.

Publications

The following publications have been published or submitted, and include a reference to the project in the Acknowledgement section: "Research supported by the Leibniz Competition 2014".

Publications in refereed journals

1. de Jonge, N., Theory of the spatial resolution of (scanning) transmission electron microscopy of nanoparticles embedded in liquid or ice layers. ACS Nano, submitted, 2017.
2. Dahmke, I. N., Verch, A., Hermannsdörfer, J., Peckys, D.B., Weatherup, R., Hofmann, S. & de Jonge, N., Graphene liquid-enclosure for single-molecule analysis of membrane proteins in whole cells using electron microscopy. ACS Nano, under review, 2017.
3. Verch, A. & de Jonge, N., The spatial resolution of scanning transmission electron microscopy as function of vertical position within a sample. Microsc. Microanal., under review, 2017.
4. Peckys, D.B., Stoerger, C., Lattab, L., Wissenbach, U., Flockerzi, V. & de Jonge, N., The stoichiometry of the TMEM16A ion channel determined in intact plasma membranes of COS-7 cells using liquid-phase electron microscopy. J. Struct. Biol. 199, 102-113, 2017.
5. Peckys, D.B., Korf, U., Wiemann, S. & de Jonge, N., Liquid-phase electron microscopy of molecular drug response in breast cancer cells reveals irresponsive cell subpopulations related to lack of HER2 homodimers. Mol. Biol. Cell, early online, 2017.
6. Hermannsdörfer, J. & de Jonge, N., Studying Dynamic Processes of Nano Sized Objects in Liquid using Electron Microscopy. JoVE 120, e54943, 2017.
7. Dahmke, I. N., Verch, A., Weatherup, R., Hofmann, S. & de Jonge, N., Graphene enclosure facilitates single-molecule analysis of ErbB2 receptors in intact, hydrated eukaryotic cells by electron microscopy. Microsc. Microanal. 23(S1), 1304-1305, 2017.
8. de Jonge, N., Peckys, D. B., Wiemann, S. Single molecule and single cell analysis of HER2 receptors in breast cancer cells using liquid phase scanning transmission electron microscopy. Microsc. Mcroanal., 23(S1), 1106-1107, 2017.
9. de Jonge, N., Wang, C. & Ross, F.M., Transmission electron microscopy of specimens and processes in liquid. MRS Bulletin 41, 791-799, 2016.
10. Peckys, D.B., Alansary, D., Niemeyer, B.A. & de Jonge, N., Visualizing quantum dot labeled ORAI1 proteins in intact cells via correlative light- and electron microscopy. Microsc. Microanal. 22, 902-912, 2016.
11. Hermannsdörfer, J., Tinnemann, V., Peckys, D.B. & de Jonge, N., The effect of electron beam irradiation in environmental scanning transmission electron microscopy of whole cells in liquid. Microsc. Microanal. 22, 656-665, 2016.
12. Verch, A. & de Jonge, N., Depth dependence of the image resolution in scanning transmission electron microscopy experiments. Microsc. Microanal. 22(S3), 802-803, 2016.

13. Dahmke, I. N., Hermannsdörfer, J., Weatherup, R., Hofmann, S., Peckys, D. & de Jonge, N., Electron microscopy of single cells in liquid for stoichiometric analysis of transmembrane proteins. Microsc. Microanal. 22(S5), 74-75, 2016.
14. Peckys, D. B., Korf, U., Wiemann, S. & de Jonge, N., Role of heterogeneity in cancer cells examined through quantitative analysis of single HER2 protein distribution and activation status. Microsc. Microanal. 22(S5), 26-27, 2016.
15. Peckys, D.B., Korf, U. & de Jonge, N., Local variations of HER2 dimerization in breast cancer cells discovered by correlative fluorescence- and liquid electron microscopy. Science Advances 1:e1500165, 2015.
16. Peckys, D.B. & de Jonge, N., Studying the stoichiometry of epidermal growth factor receptor in intact cells using correlative microscopy. JoVE, e53186, 2015.
17. Hermannsdörfer, J. & de Jonge, N., Radiation Damage of Biological Specimen in Environmental Electron Microscopy. Microsc. Microanal. 21(S3), 891-892, 2015.
18. Peckys, D.B., Hermannsdörfer, J., Tinnemann, V., Korf, U. & de Jonge, N., Visualizing the Distribution and Stoichiometry of Growth Factor Receptors in Intact Cells in Liquid Phase with Correlative Fluorescence and Scanning Transmission Electron Microscopy. Microsc. Microanal. 21(S3), 213-214, 2015.
19. Verch, A., Hermannsdörfer J. & de Jonge, N., The Stability of Gold Nanoparticles in Liquid Scanning Transmission Electron Microscopy Experiments Studied under Varied Conditions. Microsc. Microanal. 21(S3), 1125-1126, 2015.

Book chapters

1. Peckys, D.B. & de Jonge, N., Liquid STEM for studying biological function in whole cells. in: Liquid Cell Electron Microscopy. ed. Ross, F.M., Cambridge University Press, 2016.
2. de Jonge, N., Liquid phase experiments – Describing experiments in liquids and the special requirements and considerations for such experiments. in: Controlled atmosphere transmission electron microscopy. ed. Hansen T.W. & Wagner, J.W., Springer, 2016.
3. de Jonge, N., Pfaff, M. & Peckys, D.B., Practical aspects of transmission electron microscopy in liquid. Adv. Imag. Electr. Phys. 186, 1-37, 2014.

Data handling and dissemination of results

The development of software algorithms, the simulation of data, and the data analysis has been documented according to highest scientific standards. All microscope images and other data are stored on servers at the INM with automated backup in their original format for at least 10 years. The images have only been modified in brightness and contrast according to the international standards of microscopy. Every other change to the images (raw data) has been documented, explained in the corresponding publication, and the resulting images have been stored under a new file name. The experimental protocols have been documented either in paper logbooks or electronic logbooks. All personnel involved in the project has been briefed on the correct scientific and ethical practices. All research results have been published in refereed scientific journals or are under review, and so have become available to the scientific community and beyond.

Press

1. DKFZ press release: "Mögliche Ursache für Resistenz gegen Brustkrebs-Therapie entdeckt", 20.7.2015
2. INM press release: "Researchers discover a possible reason for drug resistance in breast tumors", 15.7.2015 - visible at EurekAlert, Science Daily, MedicalXpress, etc.
3. Newspaper article: Spähprogramm für Körperzellen -Saarbrücker Wissenschaftler untersuchen mit einer neuen Technik die Zell-Kommunikation, Saarbrücker Zeitung, 4.6.2014
4. INM press releaset: "Nanopartikel helfen bei der mikroskopischen Erkennung eines bei Krebs beteiligten Eiweißmoleküls" 27.2.2014