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Correlative microscopy reveals abnormalities in type 1 diabetes

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Chapter 1

General introduction and thesis outline

The use of microscopes to visualize the unimaginable many molecular processes within cells and tissues unable to be seen by the naked eye has been key for life sciences over the past centuries. Fluorescence microscopy enables to identify specifically labeled molecules to study their respective roles in the regulation of life in health and disease. The development of fluorescent proteins (FPs), such as green fluorescent protein (GFP), opened up a new dimension, since specific proteins tagged with a FP could be imaged in time and space in living cells¹⁻³. However, the diffraction limit of fluorescent light microscopy restricts the lateral resolution to a maximum of approximately 250 nm, precluding the ability to image single biomolecules, typically ranging from 0.1 to 10 nm in size. Super-resolution fluorescent microscopy techniques have been developed to break the diffraction limit approaching the biomolecular scale⁴. However, all fluorescent microscopy approaches are based on imaging of specifically labeled molecules, leaving all the unlabeled molecules 'in the dark'⁵. With electron microscopy (EM) cellular ultrastructure can be analyzed at high resolution. However, specific molecules are hard to define in grey-scale EM data. Furthermore, EM is performed on fixed samples, precluding dynamic imaging as with FPs, making it very difficult to find rare events in time and space. Limitation of both microscopy modalities can be overcome when combined, called correlated light and electron microscopy (CLEM)^{6-8, 8}. Dynamic fluorescent imaging can directly be followed by EM to put the process in a ultrastructural context^{9, 10}. Furthermore, color introduced by fluorescence to the ever expanding high content EM techniques, such as 3D and large-scale 2D approaches, aides to analyze gigabytes of otherwise grey-scaled data¹¹⁻¹³.

Successful CLEM experiments depend on the choice of suitable probes. Correlating data for CLEM can be performed by 'simply' overlaying fluorescence images on the same region acquired with EM to analyze the ultrastructure under the diffraction limited fluorescent spot¹⁴. Therefore, all available fluorescent probes can in principle be used in CLEM experiments. Often for CLEM, a more precise localization of the targeted molecules is desired, for example by correlating super-resolution fluorescence microscopy with EM¹⁵⁻¹⁷. Moreover, by combining fluorescence with electron dense EM probes even higher resolution localization can be obtained, for example by sequential labeling of FPs by immunogold¹⁸ or by probes with both fluorescent and electron dense properties^{10, 19-21}. The recognition of electron dense probes in EM still depend on grey-scale and discrimination is mostly based on size and shape, for example with different nanoparticles. Recently, the ability to create high resolution color- instead of grey-scaled images using the primary electron beam in a scanning EM (SEM), has been explored. In this thesis, these 'colorEM' approached are either based on photon emission by specific probes, cathodoluminescence (CL)²²⁻²⁵, or the release of element specific X-rays, energy dispersive X-ray spectroscopy (EDX)^{26, 27}, upon electron beam induction, which can then be detected with an optical objective or a dedicated EDX detector respectively. Studying life sciences often involves the regulation and interaction of different molecules in time and space. Therefore, expansion of the still limited CLEM probe toolbox is highly desired to aim for multi-target color labeling with localization at high resolution.

Probes used for microscopy typically contain genetically-encoded, e.g. FPs, or affinity-based targeting to identify proteins of interest. Most affinity-based labeling approaches often involve antibody-mediated immunolabeling, which is mostly applied on fixed material, since antibodies have to penetrate cells and tissue to label the molecules of interest. However, immunolabeling does allow

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probing of endogenous molecules in (human) tissue, which is impossible for genetically-encoded targeting.

The aim of this thesis is to develop novel affinity-based CLEM and colorEM probes together with improving labeling approaches for existing probes. Next, improved probes and sample preparation approaches are implemented to study type 1 diabetes (T1D) pathology.

Outline of the thesis

Over the past decade CLEM has been boosted by the development of dedicated probes, specialized sample preparation protocols, improved image registration, dedicated microscopes, and increased throughput. Different methods and CLEM approaches emerged from a wide variety of biological questions. Therefore, there is not just one generic CLEM method for each specific research question. **Chapter 2** provides an overview of the latest developments and future directions of CLEM methodology⁶. Moreover, guidelines, tips and tricks are described to aid novel users to choose the most suitable CLEM approach for their own specific research question.

Classical transmission EM (TEM) imaging on ultrathin sections at high resolution is limited to a micrometer field of view (FOV), leaving out the ‘big picture’ context of the complete cell or tissue. Large-scale scanning transmission EM (STEM) allows acquisition of complete tissue cross sections at high resolution, termed nanotomy for nano-anatomy, generating ‘google-earth’-like datasets. Identification of cells and structures in these gigabyte grey-scaled datasets can be achieved by post-embedding immunolabeling with quantum dots (QDs) as described in **Chapter 3**²⁸. QDs showed a tenfold increased labeling efficiency compared to conventional immunogold labeling. Furthermore, QDs are both fluorescent and electron dense, making them very suitable for CLEM as shown in figure 2a of chapter 2. In addition to the ability to perform large-scale EM, STEM imaging provides sufficient contrast when heavy metal staining with uranyl acetate and lead citrate is omitted, which is required for TEM. The absence of these heavy metals facilitated the recognition of QDs on insulin granules, which were masked otherwise.

Chapter 4a and 4b discuss two electron beam induced colorEM approaches. First, **Chapter 4a** describes the use of fluorescent nanodiamonds (FNDs) as multi-purpose microscopy probes²⁹. FNDs are used for prolonged fluorescence imaging for their photostability and. Furthermore, nitrogen vacancy containing FNDs have the potential to be observed with CL. Therefore here, CL experiments were performed on smaller FNDs, i.e. 40 and 70 nm, in both an uptake assay and pre-embedding immunolabeling, followed by high resolution localization in epon sections.

Chapter 4b³⁰. When performing EM, the focused primary electron beam may eject inner shell electrons from the atoms within a sample, creating an electron vacancy. Then an electron from an higher energy outer shell fills the vacancy and the difference in energy is released in the form of an X-ray. The energies of the emitted X-rays are element specific. By equipping a SEM with a dedicated EDX detector, the elemental content per scanned pixel can be fingerprinted and reconstructed to color images at EM resolution (‘colorEM’). EDX allowed to discriminate between 10 nm immunogold labels and cadmium containing QDs, which are otherwise difficult to distinguish. Furthermore, different cell types within rat islets of Langerhans could be discriminated in a label-free manner by looking at the endogenous elements present in the secretory granules. Insulin granules for example are specifically enriched in sulfur. Interestingly, EDX identified endocrine cells at the border of the islet of the diabetic prone normoglycaemic rat used for the method development also contained granules from the exocrine pancreas together with an overall affected ultrastructure. This

observation may hint to a harmful interaction between exocrine and endocrine cells at the onset of T1D.

The underlying mechanism(s) initiation beta cell destruction resulting in type 1 diabetes (T1D) are still poorly understood. Understanding the T1D etiology demands full knowledge of cellular composition and microenvironment of the islets of Langerhans to ultimately find alternatives for insulin therapy. **Chapter 5a** describes an online nanotome repository of human T1D pancreatic tissue from the network of pancreatic organ donors (nPOD). A first round of analysis of the database revealed the presence of atypical immune cells specifically present in T1D pancreases. Moreover, with EDX intermediate endocrine-exocrine cells were identified in the islets of two of eight T1D donors, and not in non-diabetic donors, similar as observed for rat tissue in chapter 4b. Since intermediate cells have been observed in early-onset T1D human donor islets and in 'pre-diabetic' rats, we hypothesized that exocrine cell damage triggers beta cell stress at T1D onset. This is in accordance with a current notion that the complete pancreas rather than only the islets of Langerhans is affected during T1D. Findings from pilot functional follow up experiments to elucidate beta cell stress upon an exocrine cell trigger are discussed in **chapter 5b**. Insulinoma cell lines have been stimulated with exocrine cell lysates to subsequently assess a possible T1D associated stress response. Most interesting a chemokine that is a prominent expressed during early onset T1D³¹ (CXCL-10) is increased by a rat insuloma cell line specifically upon treatment with exocrine cell lysates. This indicates a pro-inflammatory response by these cells. Taken together, EM/EDX data from chapters 4b and 5a combined with findings from the functional follow up experiments from chapter 5b might indicate that exocrine cell damage might serve as a trigger to T1D. Furthermore, Chapter 5b discusses possible follow up experiments and model optimization possibilities to investigate the role of the exocrine pancreas at the onset of T1D.

Chapter 6 provides a summary and general discussion of the thesis. Furthermore, future perspectives following the work in this thesis are discussed. I here conclude that CLEM is a powerful approach to combine the strengths of both light microscopy and electron microscopy modalities. CLEM probes ideally contain fluorescent and electron dense properties to visualize them both in overview, using fluorescence microscopy combined with large scale EM, and to localize them at high resolution in the context of ultrastructure. To expand the CLEM probe tool kit the electron beam will get a prominent role, like with CL and EDX. To achieve multi-target CLEM by affinity based labeling of endogenous targets in (human) tissue, immunoEM approaches need to be further optimized and standardized. By combining the large-scale EM method nanotome with visualizing endogenous molecules using immunoEM and EDX provided novel clues for a possible trigger for type 1 diabetes, which earned a functional follow up granted by the *European association for the study of diabetes* (EASD). Overall, the take home message is that novel important insights into disease mechanisms or normal life regulation can be gained by combining newly developed advanced microscopy techniques.

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