Chapter 6

Summary, general discussion and perspectives

Summary and discussion

Implementation of various microscopy techniques has been essential to study the regulation of life and disease for many years. Continuously ongoing development of hardware, software, molecular targeting etc. enables to see an increasing amount of molecular features at high resolution. CLEM utilizes the strengths of both fluorescent light- and EM to complement their respective limitations. LM is used for specific and effective molecular targeting, possible followed by live cell imaging, and provides color to grey-scaled EM. Subsequently, EM provides high resolution context to data recorded with LM. **Chapter 2** reviewed recent CLEM developments on sample preparation, data acquisition and correlation, dedicated probes, and future perspectives[1]. Furthermore, guidelines with tips and tricks, including a literature overview of past performed CLEM applications (chapter 2 table S1), for novel user is provided. As many CLEM approaches are possible, choice of CLEM is primarily guided by the kind and size of material and the specific research question. Furthermore, CLEM experiments depend on whether pre- or post-embedding labeling applies, the possibility for genetically encoded tags, epitope recognition, and microscope availability. Since many specialized and often expensive equipment exist, such as integrated microscopes, dedicated sample holders, 3D EM microscopes etc., for a novel user with a more complicated research questions it is advisable to collaborate with experienced laboratories. However, more straightforward approaches can also be sufficient by using standard confocal microscopes and EM equipment available at most research institutes combined with regular labeling strategies and sample preparation.

For many years a large gap between the imaging scales of LM and EM existed. With the development of super-resolution fluorescent microscopy LM scales are approaching EM[2, 3]. On the other hand, a recent boost in development of high content 3D and 2D EM techniques bridges EM further to LM scales[4, 5]. Classical transmission EM (TEM) on ultrathin sections at high resolution is restricted to a field of view of a few micrometers leaving out the complete cellular or tissue context. With large-scale scanning transmission EM (STEM), complete tissue cross sections can be recorded for 'google-earth'-like, post-acquisition analysis, called nanotomy for nano-anatomy[6, 7] which is shared open access upon publication. However, the vast amount of grey-scaled data recorded with nanotomy is hard to analyze. The aim here was to introduce color to these datasets using affinity based probes to label endogenous targets in (human) tissue which is impossible with genetically encoded tags.

Identification of cells and structures can be achieved with post-embedding immunolabeling using quantum dots (QDs) such as described in **Chapter 3**[8]. Post-embedding immunolabeling provides a superior ultrastructure preservation compared to pre-embedding labeling, since permeabilization required for pre-embedding labeling strongly affects the ultrastructure[9]. Furthermore EM visible nanoparticles have a low penetration depth in cells and tissue[10]. However, in most cases immunolabeling on epon sections as performed in chapter 3 is often unsuccessful since epitopes are masked and affected by strong fixation and the resin. Only about 10% of the tested targets showed positive labeling (data not shown). Post-embedding labeling probably depends in part on the concentration of the target molecules, for example insulin and amylase are present at high concentrations in their respective secretory granules and successfully labeled. Adapted sample preparation might increase labeling efficiency, such as osmium-free embedding, harsh etching, or embedding in slightly hydrophilic resins[11-13]. However, these approaches again often affect the

ultrastructure preservation. Therefore choosing a method for immunoEM is a balance between efficiency and preservation. The golden standard for immunoEM is the Tokuyasu method using thawing of cryosections[14]. However, homogenous cryo-fixation, required for Tokuyasu, of bigger tissue sections is often not possible.

Integrated LM and EM in one microscope, such as the SECOM (Delmic; Delft, The Netherlands), have been developed to facilitate high precision CLEM overlays and circumvent possible damage from sample transfer between microscopes[15]. Integrated CLEM requires fluorescent EM samples, which is usually quenched by osmium during EM sample preparation. Protocols have been developed for fluorescence preservation of FPs in cultured cells[16, 17]. However, post-embedding immunolabeling with fluorescent probes can also be used for labeling of endogenous proteins in tissue sections[18].

Post-embedding immunolabeling with QDs showed a tenfold increased labeling efficiency compared with the more classically applied immunogold (**Chapter 3**)[8]. Possibly because of a strong steric hindrance of gold particles[19]. Furthermore, QDs are both fluorescent and electron dense, therefore very suitable for CLEM purposes (chapter 2 figure 2a)[1]. QD contrast is less than immunogold and are therefore most times masked by tissue contrast when heavy metal staining is applied. When uranyl acetate and lead citrate staining is omitted QDs are well visible. However, uranyl and lead are required for proper TEM imaging. With STEM on the other hand, acquisition contrast is sufficient in the absence of heavy metal staining, making it possible to identify QDs within a proper visible ultrastructure.

The availability of CLEM probes such as QDs is rather rare, since most probes are either fluorescent or electron dense, or too large for high precision targeting. The requirement of electron density can be circumvented by inducing photon emission upon electron beam excitation, cathodoluminescence (CL), by specific probes which can then be detected with the SECOM. Subsequently, the photon originating spot can be localized at nanometer resolution. Fluorescent nanodiamonds (FNDs) are CL potent nanoparticles. In **Chapter 4a** high resolution CL from FNDs in ultrathin epon sections was detected after phagocytosis and immunolabeling upon streptavidin conjugation of the particles[20]. Thus far, most CL studies were performed using large 100-150 nm sized FNDs and in uptake assays only in a biological context[21, 22]. Although FNDs showed potential as a novel CLEM probe, they still sized in a 40-70 nm range, compared to 10-15 nm QDs, and have a high polydispersity. Furthermore, immunolabeling efficiency of streptavidin-conjugated FNDs was low compared to QDs. However, these first results might provide an avenue for further optimization for FND CLEM applications.

In **Chapter 4b** another approach to introduce color using the primary electron beam was described called electron dispersive X-ray spectroscopy (EDX)[23]. When interacting with a sample, the primary electron beam may eject inner shell electrons from the atoms present creating an electron vacancy. An electron from a higher energy outer shell then fills the vacancy and the difference in energy is released as an element specific X-ray. By adding a special EDX detector to a SEM the elemental content per pixel can be fingerprinted and translated to color image with EM resolution ('colorEM'). The application of EDX in life sciences has been limited since EDX detectors lacked sensitivity to discriminate elements in biological samples which mainly consist of carbon. The use of a high-sensitive silicon drift detector (SDD)[24] with high current in a high resolution SEM now enables to determine subcellular variations in elemental composition at the nanoscale in ultrathin biological samples. Here nanoparticles with different elemental composition, nanogold and cadmium containing QDs, could be discriminated upon post-embedding labeling. Furthermore, subcellular structures could be discriminated based on enrichment of endogenous elements such as sulfur in insulin granules since insulin contains a high number of cysteines. Moreover, different cell types in rat islets of Langerhans could be discriminated based on specific granular elemental enrichment.

ColorEM was pioneered on islets of Langerhans of a normoglycaemic diabetic prone BioBreeding (BB) rat, a model for type 1 diabetes (T1D). Unexpectedly, colorEM combined with post-embedding immulabeling revealed endocrine cells at the islet's border contained additional granules from the exocrine pancreas accompanied with an overall affected ultrastructure. These intermediate cells are solely observed in islets of diabetic prone and not the control diabetic resistant rat (unpublished data), which may hint to a harmful interaction between exocrine and endocrine at the onset of diabetes in BB rats.

The mechanism(s) initiating autoimmune destruction of insulin producing beta cells in the islets of Langerhans resulting in T1D are still poorly understood precluding alternatives for insulin therapy. Knowledge of cellular composition and the islets' microenvironment under both T1D and non-diabetic conditions is key to understand T1D etiology. Nanotomy has been pioneered in the BB rat model for unbiased analysis of T1D pathology at the nano scale during different stages of the disease[5]. Although different T1D animal models provided invaluable information about autoimmune mediated beta cell death, there are major discrepancies between animal model and human T1D pathogenesis[25, 26]. Therefore, an online nanotomy repository of human T1D pancreatic tissue from the network of pancreatic organ donors (nPOD) has been created, as described in **Chapter 5a**. The repository will become open access upon publication and datasets are coupled to a specific donor number. Although the limited datasets per donor makes the repository less suitable for quantitative analysis, a qualitative survey on specific subcellular alterations can have great added value to findings with other assays by other nPOD researchers.

T1D is considered as a T cell driven autoimmune disease[27]. A first analysis of the nPOD nanotomy database revealed to presence of unexpected immune cells, including a mast cell subtype specifically present in T1D donor tissue. Upon further inspection of the database combined with EDX analysis endocrine-exocrine intermediate cells in islets of two out of eight T1D donors, and not in non-diabetic donors, were identified. 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 line with a current growing notion that the exocrine pancreas, and not only the islets of Langerhans, is affected during T1D[28]. Patients have a significantly reduced pancreas while islets compose just a few percent of the whole pancreas, and exocrine insufficiency has been reported[29-31]. Moreover, a decreased pancreas weight was already observed in autoantibody positive donors, known to have maintained beta cell mass, indicating that exocrine tissue might be affected before changes of islets in T1D[32]. A functional follow up pilot, described in **chapter 5b**, upon static EM experiments suggest pro-inflammatory response by beta cells after exocrine stimulation. Insulin producing cell lines showed increased mRNA expression of the C-X-C motif chemokine ligand 10 (CXCL-10) upon treatment with exocrine cell line lysates compared to both a negative control and beta cell lysate treatment. CXCL10 is a potent chemoattractant for T cells[33] and its expression is strongly associated with early onset T1D[34]. Furthermore, beta cells have been identified as a possible resource for CXCL10 during T1D[35, 36].

Future perspectives

For multi-target CLEM labeling, i.e. color and high resolution localization, it is desirable to search beyond regular fluorescence for expansion of the usable color pallet and discriminate the probes at high resolution. Electron beam induced methods like CL and EDX expand the available colors visible at high resolution[20, 23]. Since CL is still photon based it's detection and discrimination is still based on the spectrum of light and thereby availability of filters. However, parameters such as CL lifetime upon

electron irradiation have already been explored to define specific nanoparticles in CL[37]. Another limitation of most nanoparticle probes used for CL is size, such as the relatively large and polydisperse FNDs used in chapter 4a[20]. Upon valuable proof-of-principle work for CL in biological samples, the next step is to develop smaller and monodisperse particles.

With EDX, probe development is not limited anymore to the spectrum of light and particle shape in EM for high-resolution discrimination and localization. In principle the periodic table of elements can be used, of which a proof-of-principle was shown for immunogold and cadmium-based QDs in chapter 4b[23]. In addition, heavy metal contrasting such as osmium, uranyl, lead, and addition stains can be used, comparable with eosin and hematoxylin in histology, upon definition of specific molecules they bind. Interestingly, defining structures and molecules with EDX can be performed based on endogenous elements, i.e. in the absence of probes, such as done for nitrogen, phosphor and sulfur based discrimination of cells in chapter 4b[23]. Moreover, optimized sample preparation protocols to prevent wash-out of elements and possibly improved detector sensitivity can aid to visualize the localization of more difficult elements such as iron, zinc, sodium etc. EDX has a long history material sciences and application and development for life sciences is still in it's infancy. Upon improvements in probe development, sample preparation and last but not least dedication from industry I predict a bright future for EDX imaging in life sciences.

ImmunoEM is limited in the number of targets and sample preparation is often a balance between ultrastructure and antigenicity preservation (discussed above). More generic immunoEM protocols would greatly benefit multi-target labeling with the potential of novel probes as discussed above. However for now, empirically testing of antibodies and exploring different sample preparation and labeling protocols, such as epon versus Tokuyasu or high pressure freezing followed by freeze substitution, is required for immunoEM. Moreover, novel CLEM probes and colorEM methods can be implemented in successful multi-target labeling protocols which otherwise use hard-to-dicriminate probes, such as different sized gold particles. In these cases it is not so much changing, but improving a winning team.

Nanotomy combined with successful post-embedding immunolabeling and EDX revealed the presence of intermediate exocrine and endocrine cells in islets of Langerhans of 'pre-diabetic' rats and early onset T1D donors (chapters 4b and 5a)[23]. The following hypothesis that damaged exocrine cells might serve as a trigger to T1D received a functional follow up study granted by the *European association for the study of diabetes* (EASD). Pilot experiments using cell lines suggested a beta cell specific response upon exocrine cell damage (chapter 5b). However, the cell line based model used is rather poor. Therefore, experimental models better controlling exocrine cell damage and reflecting a T1D beta cell response are required. Rodent or human isolated islets could replace the cell lines. Furthermore, induced pluripotent stem cell (iPSC) derived beta cells can be created and used as target cells in case of isolated islet shortage. Next, expression of a photosensitizing fluorescent protein in exocrine cells could selectively ablate these cells in co-culture with either cell lines, isolated islets or iPSC-derived beta cells. Moreover, beta cell stress upon exocrine-endocrine interactions could be studied in vivo by photosensitization induced exocrine cell damage in the vicinity of islet beta cells in a model organism such as the zebrafish.

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