Plenary Lectures abstracts

Emerging fluorescence technology to study the spatial and temporal dynamics of organelles within cells

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Emerging visualization technologies are playing an increasingly important role in the study of numerous aspects of cell biology, capturing processes at the level of whole organisms down to single molecules. While developments in probes and microscopes are dramatically expanding the areas of productive imaging, there are still significant roadblocks. Primary challenges include 1) fluorophore bleed-through, which limits the number of fluorophores that can be simultaneously imagined, 2) imaging speeds that are too slow, and 3) labeling densities that are too low for deciphering fine subcellular architecture. Here, I will discuss new imaging methods that can overcome these roadblocks, focusing on their potential for clarifying subcellular organelle dynamics. To surmount fluorophore bleed-through, we combined excitation-based spectral unmixing and lattice light sheet microscopy to visualize up to six organelles (i.e., ER, Golgi, mitochondria, lysosomes, peroxisomes and lipid droplets) simultaneously within cells. This allowed us to track these organelles through time and analyze their inter-organelle contacts. To increase temporal resolution during imaging, we employed total internal reflection fluorescence combined with structured illumination microscopy to visualize organelle dynamics at very high temporal-spatial resolution. Examining the ER, we observed that many peripheral ER sheets seen using diffraction-limited imaging are actually highly perforated structures comprised of tightly packed groups of dynamic tubules. Within the latticed ER tubule meshwork, subdiffraction-limited holes were observed (~150-250 nm diameter) having transient lifespans (~250 msec). Viewed at higher resolution using lattice light sheet microscopy combined with point accumulation for nanoscale topology (PAINT), the peripheral ER sheets represented a complex meshwork of tightly cross-linked ER tubules. I discuss possible roles this complex ER structural organization has for diverse cellular functions.

New insights into the regulation of mammalian meiosis revealed by Structured Illumination Microscopy

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During meiosis, cohesion between sister chromatids (SCC) and synaptonemal complex (SC) formation are mediated by ring-shaped protein complexes called cohesin complexes. Correct establishment of SCC and SC formation ensures that chromosomes are correctly segregated into viable gametes. The SC has an intricate ladder organization where transverse filaments connect a ~25 nm wide central element and two lateral elements ~100 nm apart. By employing structured illumination microscopy, we have shown that in order for the SC to correctly assemble between homologues chromosomes, a distance smaller than 15% of a chromosome length must be maintained between adjacent cohesin complexes. Maintaining cohesin complexes at a short distance prevents a local separation of chromosome axes and consequently, illegitimate SC formation between sister chromatids [1,2]. We have recently found that a significantly narrower SC structure is present in female mice, when compared to the SC of male mice. We are currently employing a combination of super-resolution and electron microscopy techniques, as well as biochemistry, in order to pin point which component(s) of its ladder-like structure differ between the two sexes. A recent development of 3D-multi focus-SIM and its application to the study of SC will be introduced by Dr. Sara Abrahamsson. We will present and discuss our findings and their possible implications to human fertility.

Project Discovery: How 200,000 gamers contributed to the Human Protein Atlas

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Understanding protein localization is one of the keys to understanding protein function and cellular biology. The Cell Atlas aims to discover these locations on a proteome wide scale through high-resolution confocal microscopy. To analyze the massive amount of data created in this undertaking we turned to an unlikely source, video gamers. By partnering with MMOS and CCP games we were able to leverage an existing massive multiplayer online gaming community in EVE online, to quickly analyze massive quantities of high-resolution confocal microscopy images within a mini-game we call “Project Discovery”. The game was launched in March 2016 and to date over 200,000 players contributed with over 20 million image classifications. Through their work, players have refined protein localizations on the Cell Atlas. Project Discovery has become one of the most successful implementations of citizen science ever and all resulting analysis is publicly available as part of the Human Protein Atlas’ Cell Atlas.

Multi-dimensional imaging during plant cell differentiation

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The development of higher organisms depends on the tight spatio-temporal regulation of cell dynamics, enabling stem cells to become specialized cells in specific tissues. This cellular differentiation process corresponds to a chronological sequence distinct for every cell-type, in which specific successive changes alter the cell to acquire a specialized shape, metabolism and function. However, the exact sequence as well as the duration and progression of each step still remain unclear. Moreover, the location of certain cell-types in internal tissues hinders their non-destructive accessibility. To therefore assess the spatio-temporal behavior of cell differentiation present in internal tissues, we developed inducible pluripotent stem cell cultures (iPSCs). These allow the synchronized production on-demand of specific cell-types, such as vascular cells, in response to specific growth factors. These iPSCs were developed in plants which have unique cellular features, such as the presence of cell walls that protects and immobilizes every cell in the organism. In parallel, we optimized novel imaging methodologies including real-time live cell imaging and microspectroscopy to observe and quantify the cell dynamic at a high resolution during long time periods. Combining these imaging approaches together with genetic engineering and pharmacology, we were able to decipher the sequential steps followed by a stem cell to become a plant vascular cell. These specific changes included the reorganization of the cytoskeleton to spatially dictate the formation of a secondary cell wall, followed by programmed cell death and then several specific post-mortem modifications. Altogether, our approaches and methodologies enabled breakthroughs in understanding the dynamic differentiation of plant vascular cells.

Cryo-EM Imaging of the Unimaginable Complexity of Protein Synthesis at Atomic Resolution

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Cryo-EM has been revolutionising the field of biomaging by producing accurate atomic models of central macromolecular complexes involved in key cellular pathways. We applied cryo-EM for imaging the elusive protein synthesis machineries in human, yeast and plant organelles: mitochondria and chloroplasts. The generated atomic models of mitoribosomes and chlororibosomes revealed extreme diversity of the translational apparatus addressing specific requirements of the corresponding cell’s compartments. The new structural gallery explains not only how the protein synthesis in organelles is orchestrated, but also provides a glimpse into the evolutionary processes driving the design of specialised ribosomes.
Electron cryomicroscopy for in situ structural biology

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I will present recent results from the laboratory to illustrate how we used modern electron microscopy methods to conduct high-resolution structural and cell biology studies. We have combined in vitro reconstitution with sub-nanometre resolution in vivo imaging to solve structures of bacterial cell surface proteins. We have also applied correlated cryogenic light and electron microscopy to locate target molecules in crowded cellular environments within cells. These results show the potential of using in situ structural biology to solve structures of macromolecules in their native environments.

Current and future bio-imaging opportunities at synchrotrons.

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Visualizing fast micrometer-scale internal movements in small animals, plants and bacteria is a key challenge for studies in functional anatomy, physiology and biomechanics. Traditionally, X-ray imaging holds a strong position on this field. In-vivo X-ray computed tomography is routinely performed down to a spatial resolution of several tens of micrometers [1,2]. The brightest X-ray source ever built is the MAX IV Laboratory [3] in Lund inaugurated in June 2016 to push the limits of resolution in time and space further.

We anticipate that looking into the living tissue in its native environment will be soon feasible in a true 3D manner at the micrometer scale. Three dimensional in vitro studies down to 10 nm resolution will become reality using coherent X-ray nano-imaging techniques. As the potential is significant, the expectations are accordingly high, but the road to success is not evident.

The big amount of data (often 1 TB/min already today) in the form of X-ray tomograms is visually often appealing, but the true scientific values are buried deeply in these multidimensional arrays. The tools to exploit them, interpret and visualize are scattered across the diverse scientific communities. I will highlight the success stories from the past [3,4,5] when such tools contributed significantly to the understanding of processes and the organization of living matter. At the same time I will point out where in my opinion a significant shift of paradigm is required in terms of quantification of multidimensional images.

Poster Abstracts

1.
Delicate in vitro models of vascular and cellular interactions

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The endothelial cells (ECs) that line the internal surface of the blood vessels participate in the regulation of haemostasis. Due to the crosstalk between haemostasis and the immune system, a disruption in the ECs function, caused by damage or stimulation, result in coagulation activation and an immune response. In this experimental study, a new model that enables the interaction between ECs and whole blood [1] has been used with the aim to generate positive controls for procoagulative and proinflammatory responses. Plasma samples obtained were analyzed for platelet count, TAT formation, and C5b-9 complex formation using ELISA, and for the recruitment of immune cells and platelets in fluorescence microscopy analysis. Results showed an increase in coagulation activation for ECs stimulated with LPS compared to nonstimulated ECs. A recruitment of immune cells and platelets was also seen. Additionally, a heparin conjugate (CHC) was investigated for its potential as a local anticoagulantia for the protection of ECs. The interaction between CHC and platelets was studied using FACS and results indicated that CHC bound to activated platelets rather than inactivated platelets while CHC itself neglected activation of platelets compared to the platelet agonist TRAP. Furthermore, CHC bound to ECs was seen to be taken up by the cells and degrade after 72 h. In conclusion, TGN1412 and LPS might be used as positive controls for future applications where this new model will be used to analyze biopharmaceuticals, administrated systemically, for preclinical prediction of innate immunity in vitro. Also, the results support the usage of CHC as a localized protection of the vascular endothelium in thrombotic diseases upon binding to activated platelets and ECs, where CHC degrade over time.

References

2.
Protein aggregation and chaperone dynamics during stress in Caulobacter crescentus

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All life forms have systems in place for proper folding and maintenance of their protein complement. Stresses, such as antibiotic treatments or elevated temperature, can cause native proteins to un- or misfold, which can result in the formation of toxic protein aggregates. In bacteria the chaperones DnaKJ, ClpB, GroESL, and small heat shock proteins are primarily responsible for removing misfolded proteins. These proteins have been well studied, however their in vivo responses to environmental changes are incompletely described. Additionally, systems for coping with the accumulation of toxic aggregates when the capacity of the folding machinery is exhausted have only recently begun to be investigated in bacteria.

Using a combination of fluorescence and time lapse microscopy, we study the dynamics of protein aggregation and chaperone function in the model organism Caulobacter crescentus, an asymmetrically dividing bacterium that produces two morphologically distinct daughter cells. Our results demonstrate that the formation of protein aggregates and their dissolution depends on the intensity of stress experienced. Stress induces protein aggregation visible as punctate foci throughout the cell volume, to which chaperones localize. During mild stress these foci are quickly resolved, while during more severe stress chaperone localization increases dramatically without further reorganization of the aggregates. Preliminary data suggests small heat shock proteins may play a role in aggregate size, and that chromosome topology may govern the location of aggregate accumulation.
3. rsFPs For Non Invasive Super-Resolution Microscopy

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Reversibly photo-switchable fluorescent proteins (rsFPs) allow to significantly lower the illumination intensities in super-resolution imaging techniques, such as reversible saturable optical fluorescence transition microscopy (RESOLFT) and nonlinear structured illumination microscopy (NL-SIM). The low illumination intensities needed with these techniques make them suitable for non-invasive live-cell imaging at the nanoscale. The techniques take advantage of the molecular transitions provided by rsFPs of different types and especially their positive and negative switching mechanism.

Several kinds of rsFPs have been developed during the last few years, paving the way for these novel imaging technique. Dronpa, GFP, YFP, Cherry, mMaple and mEos mutants have been used for RESOLFT, SOFI and NL-SIM imaging. However, each nanoscopy approach asks for specific measurement protocols, making it difficult to find a common framework for comparison. Here we provide a photo-physical and imaging toolbox to quantify and compare several rsFPs parameters to best perform RESOLFT imaging, both in the point-scanning and in a novel parallelized implementation. Parameters such as brightness, switching efficiency and kinetics, fluorescence lifetime, contrast between ON (fluorescent) and OFF (not fluorescent) states and last switching fatigue are quantified and compared for different rsFP families and also for mutants belonging to the same family. We take advantage of the full characterization of the rsFPs behaviour together with a new flexible Multifocal RESOLFT setup to push the limits of large field of view RESOLFT microscopy.

4. 3D STED Microscopy For Super-Resolution With Quantum Dots And Permeable Dyes

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The family of super-resolution microscopy methods based on the idea of STED is ever growing. In our lab, we are developing a new STED microscope that combines previous 2D and 3D-based methods, multi-color approaches and adaptive optics implementations, all into one microscope that operates in the near-infrared part of the spectrum. A spatial light modulator is used to generate two separate and complementary depletion patterns that simultaneously deplete the fluorescence. Moreover, a single STED color is used to deplete multiple dyes, which has been made possible through recent breakthroughs concerning suitable combinations of STED-compatible and cell permeable dyes that allow for high resolution in two colors simultaneously. To achieve the best depletion, minimize photo-damage and facilitate deeper tissue imaging all colors are in the red part of the spectrum. Together, this allows for functional nanoscale imaging with a three-dimensional spatial accuracy in the range of 30x30x70 nm. The approach is being developed to allow for live cell imaging and aim to study organelles and macromolecular complexes in living neuronal cells of rodent brain tissues.

Improving the performance of super-resolution methods can also be achieved by optimizing the choice of fluorescent labels. While dyes and fluorescent proteins are the most commonly used labels for STED microscopy today, quantum dots (QDs) have recently been shown to be a viable option in the near-infrared part of the spectrum. The unique properties of QDs, such as outstanding brightness and long-term photostability, are intriguing in the search for better fluorophores, and may open new applications to be investigated by STED microscopy. We have been able to extend the idea and have shown STED imaging of QDs with a spatial resolution improvement higher than twofold, with QDs in the more blue-shifted green-orange part of the spectrum. Moreover, through time-correlated single photon counting we have concluded that the lifetime parameters of QDs are significantly affected by excitation light intensity as well as STED illumination. This agrees with a previously published model for exciton energy level interactions in QDs at a varying excitation intensity. These results show that QDs has potential to be more widely used as fluorescent labels for standard super-resolution methods such as STED microscopy, and that novel methods based on excitation intensity manipulation and lifetime separation are viable.
5.

Cryo-EM Imaging of the Packaged Genome in the Native and Swollen Tomato Bushy Stunt Virus Particles

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Tomato bushy stunt virus was the first virus particle imaged to atomic resolution¹. Since crystal packing is determined by particle contacts, X-ray crystallography (XRC) by necessity average over all 60 icosahedral orientations. Likewise, most electron cryo-microscopy (cryo-EM) structures of virus particles have averaged over the icosahedral symmetry. This enhances the sampling tremendously, but perhaps even more importantly, orientation determination of highly symmetric objects is very difficult. Recent improvements in cryo-EM technology with direct electron detectors now allows for the asymmetric reconstruction of virus particles to near-atomic resolution. This allows for the reconstruction of the packaged genome as well as minor proteins². In this study, we purified virus particles of tomato bushy stunt virus (TBSV) from plants and imaged them by cryo-EM using a Titan Krios microscope with a Gatan K2 Summit direct electron detector and energy filter. A dataset with approximately 32,000 particles was reconstructed with symmetry averaging to 3.2 Å resolution (Fig. 1a,c). A mesh of previously unreported density was found beneath the capsid. This density likely corresponds to structured duplex RNA in associated with the N-terminal RNA-binding domain of the coat protein. An asymmetric reconstruction of the same dataset reached 4.5 Å resolution and revealed additional RNA density at lower radii (Fig. 1d). By mimicking the conditions of the cytoplasm of the host cell (by removing bound Ca²⁺ ions and raising the pH to slightly alkaline conditions), one can induce a swollen state of the virus particle. An 8 Å resolution XRC structure of this disassembly intermediate shows that the particle radius increase by ~10% and large pores form through the capsid³. We collected a dataset with approximately 6,000 particles of swollen particles and the symmetry averaged reconstruction reached 4.6 Å resolution (Fig. 1b). The structure revealed interesting structural rearrangements of the coat proteins and that the RNA cage remained associated with the capsid.

Figure 1. Reconstructions of TBSV (a) Native particle at 3.2 Å with radial color scale (capsid in red-yellow and RNA in green). (b) Swollen particle at 4.6 Å (same coloring scheme). (c) Detail of map in (a) showing a region of the capsid with two calcium binding sites. (d) Detail from an asymmetric reconstruction of the native particle showing RNA density at the center of the particle (same coloring scheme as in (a)).

6. **Functional role of v-SNAREs in secretory granule exocytosis**

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Insulin is stored in secretory granules and released by regulated exocytosis. This secretion is the result of a distinct cascade of events, including granule maturation, docking, priming, and finally Ca2+-dependent fusion. The soluble NSF attachment receptor (SNARE) proteins are the core machinery required for all intracellular membrane fusion events. While SNAREs specifically control fusion, vesicular SNAREs (v-SNAREs) perform additional roles that are still elusive. In secretory cell types, v-SNAREs are pivotal for targeting vesicles to different locations, mediating both sorting and membrane protein distribution and reductions in v-SNAREs result in defective regulated exocytosis. Using total internal reflection fluorescence (TIRF) microscopy, we studied how the v-SNARE VAMP8 effects secretory granule exocytosis in pancreatic beta cells. We found that VAMP8 associates with early, late and recycling endosomes instead of insulin containing secretory granules and that VAMP8 vesicles traffic specifically with recycling endosomes. VAMP8 expression reduced secretory granule fusion, whereas VAMP2 expression facilitated granule fusion, delineating a VAMP8-dependent fusion step between recycling endosomes and the plasma membrane. Truncation of the VAMP8 C-terminal region resulted in less attenuation of exocytosis than transmembrane containing constructs, which drastically reduced exocytosis of granules. This implies that VAMP8 may function in granule genesis and the sorting of essential membrane proteins regulating insulin secretion. Additionally, the TMD of VAMP8 may be necessary for initiating fusion and appears to have a direct interaction with the exocytosis machinery.

7. **Disturbed association of calcium-channels with insulin granules underlies secretory defects in human type-2 diabetes**

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Loss of 1st phase secretion is an early sign of developing type-2 diabetes. Calcium-entry through voltage-gated L-type Calcium-channels triggers exocytosis of insulin-containing granules in pancreatic β-cells and is required for the postprandial spike in insulin secretion. Using high resolution microscopy we identify a subset of docked insulin granules where localized Calcium-influx triggers exocytosis with high probability and minimal latency in human β-cells and clonal INS1 cells. This immediately releasable pool of granules (IRP), identified both structurally and functionally, was absent in β-cells of human type-2 diabetic donors and after long-term exposure to fatty acids that mimic the diabetic state. Upon arrival at the plasma membrane, IRP granules slowly associated with 15-20 L-type channels. This recruitment depended on a direct interaction with Munc13, because expression of the II-III loop of the channel, the C2-domain of Munc13, or of a Munc13 with a mutated C2 domain, all disrupted L-type channel clustering at granules and ablated fast exocytosis. Thus, rapid insulin secretion requires close proximity of granules with L-type Calcium-channels, which is mediated by Munc13. Loss of this organization underlies disturbed insulin secretion kinetics in type-2 diabetes.
8. Imaging the regulation of fusion pore expansion during insulin granule exocytosis

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The central feature of regulated exocytosis is formation of a fusion pore, an aqueous channel connecting vesicular lumen with the extracellular space. After exocytosis has been triggered, this complex proteo-lipidic structure acts as a molecular sieve that regulates cargo release based on size. There is evidence that dysregulated fusion pore expansion is involved in common diseases, including type-2 diabetes and Parkinson's disease. Here we have used high-resolution TIRF imaging in pancreatic b-cells to study molecular mechanisms that regulate fusion pore behavior. We screened systematically a large number of endo-and exocytosis related proteins for their presence at the release site. A pH-sensitive granule marker was used to measure fusion pore behavior in real time. The time course of GFP-labeled proteins at the release site was quantified in parallel, and the data correlated with pore behavior. A subset of membrane-curvatures sensing proteins prevented fusion pore expansion and were recruited to the release site during, or just before, exocytosis. Global analysis indicates amphipathic helixes and BAR domains as important for fusion pore restriction. At least one of these proteins has been genetically linked to type-2 diabetes.

9. Post-processing crosstalk compensation improves recall and accuracy of in-situ sequencing

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Background
Parallel sequencing of targeted RNA molecules directly in tissue samples provides a unique way to quantify gene expression without losing spatial information on tissue morphology. Investigated genes are targeted with controlled design of padlock probes, locally amplied and sequenced by repeated staining and imaging cycles in four color channels [Ke et al 2013]. Expressed genes are commonly so densely packed that image analysis of tissue at multiple focal planes becomes necessary to better separate and decode all fluorescent signals. Moreover low signal to noise ratio, chromatic aberration and signal crosstalk further complicates the analysis of multiple sequences in parallel.

Method
A cm-wide and 10 micrometer thick tissue sample acquired at seven different focal depths has been sequenced to spatially quantify the expression of 140 genes in 3D. Each RNA is uniquely identied with a five base length barcode and decoded in parallel with five rounds of sequencing according to the protocol described in [Ke et al 2013]. At each sequencing cycle, fluorescent signals were detected using a local thresholding approach based on ellipse fit [Ranefall et al 2016]. The detected signals were then aligned and combined, grouping spatially colocated signals across color channels and cycles. Image intensity values for each signal were extracted from the smoothed and dilated raw images in all color channels and normalized separately. Finally, a post-processing crosstalk compensation step was applied to the normalized intensities in order to determine the real dye concentration present in each signal. A different crosstalk compensation matrix was estimated for each of the sequencing cycles as in [Li et al 1999]. Each crosstalk compensation matrix was inverted and multiplied by the intensities of the current cycle, producing crosstalk compensated intensity values. Signal decoding was performed selecting the sequence corresponding to the highest intensity in each cycle and quality measurement for the base-calling was assessed.

Results
The implemented image analysis pipeline was able to decode more than 5000 sequences representing the expression of 140 targeted genes. Moreover, post-processing crosstalk compensation helped to consistently increase signal confidence and precision, as shown in Figure 1. Results were validated and tried out through signal evaluation with previous 2D method [Bombrun etal 2017].
Conclusion
The expression of 140 targeted genes has been quantified and spatially located in tissue using an image analysis approach to detect and decode fluorescent signals from in-situ sequencing experiments in 3D. Cross-talk compensation proved to be one of the key components at increasing the number of true positive signals as compared to unexpected false signals. Our ongoing work focuses on improving the decoding image analysis technique to provide accurate spatially resolved transcriptomics data that allows to investigate relations between RNA abundance of targeted genes and tissue morphology.

Figure 1: Number of true positive signals (TP) corresponding to detected and correctly identified barcodes against false positive signals corresponding to unknown and unexpected barcodes at increasing quality threshold levels before (red curve) and after (blue curve) crosstalk compensation. Fixing the FP/TP ratio to one over four (i.e. precision of 0.8) 4742 against 2968 true positive signals (black square markers) have been correctly decoded respectively with and without crosstalk compensation.

References

10. Image-based phenotypic profiling of patient-derived cancer cells

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Bioimage informatics is an essential part of modern biomedical research. We have been working on image-based phenotypic profiling platform that enables drug efficacy studies on patient-derived cancer cells. Our library contains 525 oncological drugs that we test in various concentrations to patient-derived cells to find the optimal treatment for a cancer patient. Recently, we have been using the platform for profiling various solid tumours such as renal and ovarian cancer. Our aim is to automate image-based phenotypic profiling for routine use in precision medicine.

Our phenotypic profiling platform includes five steps: 1) high-content imaging, 2) image preprocessing, 3) image segmentation and feature extraction, 4) supervised learning for phenotypic classification, and 5) data analysis and visualization. Quality control is included as part of every single step of the platform. We have recently developed new methods for segmentation as well as tools to train and explore the phenotypic space with machine learning models. Part of the platform is implemented in cloud environment to minimize the time needed for image processing. Our current focus is in novel supervised learning methods and data mining to find and understand previously unseen phenotypes.
11. SMLocalizer, a CUDA based ImageJ plugin for simplified PALM/STORM image analysis

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Single molecule localization microscopy (SMLM) has in the last decade been developed as an important technique for diffraction unlimited microscopy. Super resolution is achieved by a clever combination of switching fluorophore molecules and mathematical analysis software. A challenge for the non-expert user is the complexity of the computational analysis with a multitude of input parameters. Many algorithms and methods have been developed, often specific for a particular variant of SMLM.

Here we present a software solution, SMLocalizer, which reduces this complexity by automatically obtaining most of the input parameters iteratively from the data and requires minimal user input for accurate analysis. The plugin can be used to analyze 2D and the majority of published 3D modalities. The software is a plugin for the well-established ImageJ toolkit and provide a significant speed-up of analysis by use of GPU acceleration.

12. TissueMaps: A tool to visualize spatially resolved gene expression profiling data

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Spatially-resolved omics is an emerging field developing strategies to add spatial dimension to next generation sequencing (NGS). In-situ techniques performed directly on the tissue samples can achieve spatial resolution enabling detection of individual molecules in cells. In doing so, digital imaging using slide scanners creates giga-pixel images that require special methods of computerized image analysis. Cell distribution in the tissue provides a rich visual-cognitive combination of information at multiple resolutions. The lowest magnification shows specific architectural patterns in the global tissue organization. Highest magnifications provide information for profiling of gene expression at cellular resolution. Analysis at multiple resolutions opens the possibility for large-scale comparison of genotype and phenotype. Expressed genes are locally amplified by molecular probes and rolling circle amplification, and decoded by repeating the sequencing cycle for the four letters of the genetic code. Using image processing methods more than 140 genes in parallel have been identified in the same tissue sample. We have created TissueMaps, an open-source platform to automate and control the decoding process and to visualize the decoded genes. The visualization system provides interactive zooming and panning of resulting slide-scanner data with overlaid histological morphology maps, most commonly H&E staining, and DAPI staining for nuclei along with AF750 for general stain of the expressed genes. Furthermore, the user can draw regions of interest and extract local statistics on gene expression and tissue morphology at different resolutions. The decoding module uses python along with Elastix for image registration and CellProfiler (a free and open-source software developed for image based screening) while the viewer module is developed in php and javascript. TissueMaps provides a flexible solution to support the future development of histopathology, both as a diagnostic tool and as a research field.

13. A New Segmentation Method for Quantitative High-Content/High-Throughput Microscopy Analysis of Adipogenesis Models

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The capacity of cells to store neutral lipids in lipid droplets (LDs) is essential as a source of fuel for organisms. Specialized storing cells, the adipocytes, provide a buffer for energy variations. Excess accumulation or deficiency of LDs in adipose tissue
is involved in many modern-society-disorders. Intracellular LD number and size distribution reflect the tissue conditions, however, the mechanisms and genes associated with these energy reservoirs are still poorly understood. Large-scale genetic screens using human in vitro differentiated primary adipocytes require cell samples donated from many patients. The heterogeneity appearing between donors highlighted the necessity to develop high-throughput methods robust to individual variations. Previous image analysis algorithms fail to handle individual LDs, but focus on averages, hiding population heterogeneity. To develop high-content analysis (HCA) techniques for analysis of fat cell metabolism, we used data from a large-scale RNAi screen. The RNAi-based suppression of PLIN1, a protein involved in the adipocyte lipid metabolism, served as a positive control, while cells treated with randomized RNA served as negative controls. In total, 396 images of in vitro differentiated adipocytes from three donors, were acquired by an automated digital microscope. We present a new image processing algorithm for nucleus and LD segmentation and features extraction. We validated our method by extracting morphological features such as the ratio of undifferentiated cells but also the size distribution of the individual droplets per cell. K-means clustering of this feature based on the earth-mover’s distance proved to discriminate the positive PLIN1-suppressed phenotype from the untreated negative control with an area under the receiver operating characteristic curve of 0.98. The results suggest that this HCA method offers a more accurate segmentation of the individual LDs than previous methods, and can thus be utilized to quantify changes in LD metabolism in response to treatment in many cell models relevant to a variety of diseases.

**Fig.1. An algorithm able to detect the individual lipid droplets.**


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Biopsies and microscopic level analysis are essential for the diagnosis of a number of diseases. High-resolution (~1 nm) images acquired using transmission electron microscope (TEM) allows the pathologist to perform nanostructural analysis of ultrathin slice of biological sample collected from nose scraps. The utilization of TEM allows the pathologist to set the diagnosis and follow-up treatment without the need for specific stains or probes, unlike other diagnosis procedures. However, in current clinical settings, performing TEM image acquisition and analysis is manual which makes the task laborious and time-consuming. Also, the lack of skilled pathologists makes the use of TEM for routine diagnosis costly.

Currently, we are working on a project with a long-term goal to develop a generic platform to facilitate highly automated TEM imaging and analysis aid for nanostructural pathologic diagnosis. The approach includes automated microscope steering, multiscale image acquisition, and analysis. We started with one clinical application where TEM is used on a regular basis, which involves analysis of cilia cross-sections for Primary ciliary dyskinesia diagnosis. In our proposed workflow, the microscope is systematically steered and a fast search for rare biological objects of interest in a vast search space is performed in low-resolution using template based object detection approach. On detecting the sufficiently large number of objects, we refine detection results to define the region of interests (ROI) and high-resolution image set is automatically acquired for these ROI. Post-acquisition, each high-resolution image is analyzed which involves their denoising, deblurring, automatic cilia detection, registration and super-resolution image reconstruction for the enhancement of nanostructural details of cillum cross-section.
15.
Progerin expression in epidermal stem cells leads to reduced number of stem cells that divides asymmetrically and impairs Wnt signal transmission

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Hutchinson-Gilford progeria syndrome (HGPS or progeria) is a rare disease with several symptoms attributed to premature aging. Progeria is caused by a de novo point mutation in the LMNA gene. This mutation leads to a defective form of lamin A called progerin. Progerin acts by disrupting the properties and functions of the nuclear lamina, which subsequently cause premature aging. Previous studies in our lab showed that the expression of progerin in the adult stem cells of the epidermis results in impaired stratification and development of the skin. The stratification of the epidermis is a normal process during development regulated by asymmetric cell division (ACD). The stem cells of the epidermis divide asymmetrically, leading to stratification and differentiation, or symmetrically to maintain the stem cell pool. Given the observed phenotype in progeria mice we hypothesize that the expression of progerin cause deficiencies in the symmetry of stem cell division. To test this hypothesis we quantified the number of cells that divided asymmetrically vs symmetric in skin samples from progeroid and wild type mice. The results showed a significant reduction in the number of ACD. Global transcriptomic analysis of basal keratinocytes extracted from wild type and progeroid mice showed a putative role of Wnt/beta-catenin signalling in the mechanism underlying the loss of ACD in progeria. The response to pathway stimulators and segregation of this signalling components was further assayed using in vitro models. Taken together our results suggest a direct involvement of progerin in the nuclear transportation of beta-catenin and in turn, in the transmission of the Wnt signalling and normal stem cell division.

16.
Feature Augmented Deep Neural Networks for Segmentation of Cells

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In this work, we use a fully convolutional neural network for microscopy cell image segmentation. Rather than designing the network from scratch, we modify an existing network to suit our dataset. We show that improved cell segmentation can be obtained by augmenting the raw images with specialized feature maps such as eigen value of Hessian and wavelet filtered images, for training our network. We also show modality transfer learning, by training a network on phase contrast images and testing on fluorescent images. Finally we show that our network is able to segment irregularly shaped cells. We evaluate the performance of our methods on three datasets consisting of phase contrast, fluorescent and bright-field images.

17.
Novel platform for studying infiltration, migration and cytotoxicity of human Natural Killer cells in solid tumors

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Natural Killer (NK) cells represent a vital component of the immune response against tumor and are interesting candidates for cancer immunotherapy, since they do not require prior sensitization, do not induce graft-versus-host disease and are not HLA-restricted. However, NK cell-based immunotherapies to treat solid tumors have been largely unsuccessful. Here we applied a multi-well microchip platform using ultrasonic standing waves (USWs) to induce 3D tumor cell cultures for studying NK cell-solid tumor interaction with microscopy. The platform produces one hundred parallel microtumors (≈ 0,2 mm size) isolated in individual wells with an optically optimized glass bottom which allows for detailed microscopy studies. Here we made microtumors from thyroid carcinoma and renal carcinoma cells that were characterized in terms of viability and NK ligands expression. A significant modulation of MICA/B, PVR and ICAM-1 expression was observed in 3D cultures.
compared to 2D cultures, possibly caused by differences in the tumor microenvironment. From 3D co-cultures of NK cells and tumor cell we determined NK cell viability, tumor cell death and expression of NK receptors involved in development and tumor recognition. The platform is versatile allowing formation of 3D microtumors of different cell types and supports characterization of NK cell-tumor interaction by, e.g. live cell imaging, light-sheet microscopy and flow-cytometry. To make the platform useable for high throughput microscopy screenings, automatic image based characterization scripts were developed for both transmitted light images of un-stained microtumors and for confocal z-stacks of fluorescently labeled microtumors. The method could also allow the construction of microtumors with complex mixtures of cells and could, thus, help to develop strategies for personalize anti-tumor treatment.

18.
The protein-protein interaction network with functional roles in tumorigenesis, neurodegeneration, and aging

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The difficulty to resolve complex molecular mechanisms are significantly due to an involvement of multiple regulatory factors affecting the interaction networks. The studies of the interaction networks reflect the complexity better and make more sense than the traditional ‘reductionist’ point of view, which focuses only on functions of a single protein or pathway [1].

The protein-protein interaction network (PPIN) presented here, is based on the direct experimentally proven physical interactions between the proteins functionally involved in the mechanisms of tumorigenesis, neurodegeneration, and aging. The initial experimental PPIN was identified by the combination of in-situ Proximity Ligation Assay, coimmunoprecipitation, and immunocytochemistry. The intracellular visualization of the protein-protein interactions was performed using confocal laser microscopy. All the detected interacting proteins TPPII, p53, MYBBP1A, CDK2 and SIRT7 and additional interactions including SIRT6 and CD147 experimentally demonstrated by other authors, are considered as a suitable for the development of anti-tumor therapeutics and treatments for other diseases of aging [2–4]. TPPII and SIRT6 directly affect glucose metabolism which drives malignant growth. TPPII inhibitors have a decreasing effect on mTOR signalling, the enzymatic activity is required for the degradation of Aβ peptides in the human fibroblasts and it directly interacts with MYBBP1A, which is a significant activator of p53 signaling. TPPII also interacts with CDK2 [2], a protein which directly associates with proteasome subunits. Currently, SIRT7 is an intensively explored target due to the neuroprotective effects of other family members and its direct regulatory effect on mTOR signalling and autophagy. SIRT6 activators are attractive candidates for Alzheimer’s disease (AD) due to the protection effect after DNA damage. CD147 is a pleiotropic protein, which is considered as a promising target in oncological diseases, however, it also shows some impact for AD treatments [4, 5]. It should also be highlighted, that the animal and cellular models with downregulated or knockout TPPII, p53, SIRT6, SIRT7, and MYBBP1A expression levels illustrate very similar effects on the length of the life span, premature aging, and lipid metabolism.

Since the interactions involved in the aging mechanisms often have high connectivity, PPIN was further extended using the interactions from databases including both experimental and predicted interactions (Cytoscape with integrated GeneMania database, GeneAge) and by data mining of the literature. The proteins with significant roles in several diseases of aging (Alzheimer’s disease, Parkinson disease, Huntington disease, cancer) and with the effect on the lifespan are on the list of the extended PPIN. The interaction network could be potentially used for the differentiation of the mechanisms of the age-related diseases and healthy aging and for the evaluation of the side effects of treatments.

References:
19. Claudin 5-GFP transgenic reporter mouse line: A new tool to study Vascular Biology and dynamics

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The vascular system is essential to supply the body with nutrients and oxygen. The vascular system develops from scattered vascular precursor cells that create a first primitive vascular plexus. This primitive vascular plexus is later enlarged and remodeled, to create the hierarchically organized circulatory system of the adult. This requires the sprouting of new blood vessels from pre-existing ones, anastomosis of newly formed vessels, and the regression of unnecessary vascular structures. Collectively these processes are referred to as angiogenesis. Angiogenic sprouting is initiated by the formation of migratory endothelial cells (tip cells), a process driven by vascular endothelial growth factor (VEGF). VEGF binds to and activates VEGF receptors 2 and 3 (VEGFR2 and VEGFR3), which triggers angiogenic responses in tip cells, including the protrusion of filopodia that sense the environment and direct the sprout towards VEGF tissue gradients. A single tip cell spearheads the vascular sprout, whereas trailing endothelial cells (stalk cells) shape the lumenized shaft. The segregation of the sprouting endothelial cells into tip and stalk cells is regulated by Notch signaling. However, unlike many other Notch-dependent cell differentiation phenomena, which lock cell differentiation to a specific fate, the tip and stalk cell phenotypes are dynamic and reversible, as endothelial cells rapidly shuffle at the lead position. In recent years, numerous other pathways have been shown to modulate angiogenic sprouting. Although sprouting angiogenesis is a highly dynamic process – most of our knowledge comes from the analysis of fixed tissue samples. Live imaging of the vasculature in mice is technically challenging and suitable reporter tools have largely been missing. We recently developed new transgenic reporter lines that will hopefully help to overcome the technical problems and help filling existing knowledge-gaps.

20. Fast Scanning RESOLFT Microscopy for the Investigation of Live Cellular Processes

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The field of super resolution has lead the way for many great discoveries in the last few decades. However many of the techniques requires high laser powers or other harsh condition that make live cell imaging difficult and time lapse recording difficult to perform. RESOLFT is a super resolution technique that requires only a fraction of the light of for instance STED, and it does not have any requirement of chemicals that could be toxic to the cells. RESOLFT is based on reversible switchable fluorescence proteins (rsFP), and a three phase pulsing scheme ensure the super resolution. (1) Switching the rsFP into a ON, potentially fluorescent state (2) Switching the rsFP in a dark OFF state with a light pattern featuring a “zero” or multiple “zeros” of intensities (donut or line shaped) and (3) rsFPs fluorescence excitation and readout with a third light pattern to record the few rsFPs still in the ON state (REF)

RESOLFT offers many advantages for investigation of molecular process in living cells and tissue,(REF) however one caveat, especially in the point-scanning mode, is the slow acquisition speed due to the three sequential steps required, with often the off switching being the time delimiting steps. Recent years development in the rsFP, have resulted in fairly fast switching kinetics, and for instance rsEGFP2 and DronpaM159T (REF) have off switching in the micro second range, however even a faster switcher would require a recording time of about a minute to acquire a 10 by 10 μm field of view. In this work we present a new and faster scan method that can decrease the overall frame time of several factors. The principle is that instead of scanning the entire frame, we only scan objects inside the frame. The illumination beam spends a very short time to sample the pixel before it is actually recorded. During this time a quick decision is taken to “record and RESOLFT” or not the pixel. This means that only the fluorescence objects are being RESOLFT image and not the dark background. Typically in fluorescence microscopy most of you pixels are dark and this technique can therefore be used to greatly improve the time resolution.