Welcome to the 4th Winter School „Hot Topics in Molecular Imaging – TOPIM" organized by the European Society for Molecular Imaging – ESMI and the European Network of Excellence DiMI – Diagnostic Molecular Imaging. This year's hot topic is „IMAGING SYSTEMS BIOLOGY".

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welcome

Dear Participant,

it is our great pleasure to welcome you to Bardonecchia, Italy for the 4th Winter School of the European Society for Molecular Imaging (ESMI). TOPIM stands for "hot TOPics in molecular IMaging". As in previous years, our effort is to concentrate on one aspect at the forefront of Molecular Imaging, a hot topic. Since 2007, TOPIM is an annual event aiming at covering an exciting aspect of application or technique of molecular imaging every year, chosen according to its pertinence and timeliness.

Looking back at the hot topics of the previous years:

2007: Imaging in Neurodegeneration and Neuroinflammation
2008: Imaging in Nano Objects
2009: Dual and Innovative Imaging Modalities

Molecular Imaging has gained in the past years a strong influence on medicine and biology, leading the way in exploiting molecular, biological and genetic information to develop precise, precocious and predictive in vivo diagnostic methods. These methods are increasingly precious for the follow-up and the evaluation of new treatments of many pathological states. Two of the features of imaging – the bridge that it creates between biology, chemistry, physics and mathematics, and its growing importance in medicine – suffice to justify the creation of the European Society for Molecular Imaging (ESMI) with organisation of annual meetings like TOPIM. But even more crucial is the fact that imaging is asserting itself as an original means of discovery, and opens new avenues to address specifically the in vivo kinetics of disease specific molecular events. TOPIM aims at bringing scientists from various fields together and providing a "think tank" to foster new ideas and inter-disciplinary cross connections through discussions between participants. By combining expert descriptions of the most up-to-date imaging technologies and/or applications TOPIM contributes in collectively describing the imaging approaches, categorizing them and drawing the landscape of *in vivo* imaging applied to a specific scientific issue.

With the occasion of the fourth TOPIM edition, it appeared timely to engage the discussion on Imaging Systems Biology. How hot this topic for imaging scientists is could impressively be seen during our first meetings as programme committee where we discussed the various perspectives of bringing the multi-faceted phenomena studied in systems biology together with the overall goal in imaging science of how to visualise some of these aspects in the in vivo system. The effort of this winter school is to better understand uses of imaging in REAL Systems Biology problems, and/or redefine Systems Biology investigations to include the advantages of imaging, such as longitudinal readings etc. We are eager to learn what tools Systems Biology can bring to Molecular Imaging as well as what Molecular Imaging can do to integrate biological data into systems. At the same time, TOPIM is the ideal opportunity to set our seeds into our new generation of students who will hopefully bring the field even further.

We – the TOPIM 2010 programme committee have been most fortunate to attract a panel of prestigious speakers, all at the fore point of research in their discipline. We would like to thank them heartily, especially those who have travelled a long way to Italy, for having accepted to share their knowledge with us. We are really looking forward to hear about the basic paradigms of Systems Biology and their impact on Molecular Imaging and try to develop ideas on how Molecular Imaging can drive Systems Biology as well as to see demonstrations of methods to explore the biological knowledge to interpret data and to guide experiments.

We would like as well to warmly thank our sponsors, many of them being with us from the very beginning of the ESMI, for their generous support. Furthermore, we would like to point out that this year's meeting would not take place without the support and close collaboration with the European Network of Excellence DiMI – Diagnostic Molecular Imaging.

We would like to address our warmest welcome to all of you, and to encourage you to participate without restriction in the scientific debates as well as to enjoy the beauties of the mountains surrounding us.

The TOPIM 2010 Scientific Committee:

Andreas H. Jacobs, ESMI President
Clemens W.G.M. Lowik, ESMI Vice President
Bertrand Tavitian, ESMI Past President
Silvio Aime, ESMI Secretary
John Clark, ESMI Council Member
Vasilis Ntziachristos, ESMI Member

	Monday, February 8	Tuesday, February 9
08:00 - 09:00	**BREAKFAST & REGISTRATION**	
09:00 - 09:45	**Introduction SSC**	**John Lock (Huddinge, Sweden):** „A Systems Microscopy Analysis of Cell Adhesion and Migration - Applications to Novel Adhesion Biology“
09:45 - 10:30	**Opening Lecture by Hans Werner Mewes (Munich, Germany):** „Systems Biology from Knowledge Management to Images“	**Elena Kardash (Münster, Germany):** „In vivo imaging of cell migration in Zebrafish“
10:30 - 11:00		
11:00 - 11:45	**Short introductory presentation by each participant (1-2 min each)**	**Michael K. Richardson (Leiden, The Netherlands):** „Imaging of living zebrafish embryos in a microfluidics lab-on-chip“
11:45 - 12:00	**Francesca Arena (Torino, Italy):** „Poly-beta-Cycldextrin based Platform for pH mapping via a ratiometric 19F/1H MRI method“	**Eric Marijn Wielhouwer (Leiden, The Netherlands):** „Multipurpose BioChip: A novel 4D image and data acquisition technology for drug discovery“
12:00 - 12:15	**Kristine Schauer (Paris, France):** „Construction of probabilistic density maps for micropatterned cells: a computational imaging approach to systematically study the global cellular architecture of endomembranes“	**Pavel Tomancak (Dresden, Germany):** „Systematic analysis of gene expression patterns in Drosophila embryonic development“
12:15 - 12:30		**Masanari Takamiya (Karlsruhe, Germany):** „Visualization of zebrafish cornea development“
12:30	**LUNCH**	
13:30		
14:00		
15:00		
16:00		
17:00 - 17:45	**Julie Plastino (Paris, France):** „Actin and Major Sperm Protein cytoskeleton assembly for motility“	**Dimitris Kalamatianos (Maynooth, Ireland):** „Image analysis for automated live-cell imaging“
17:45 - 18:30	**Antonio Rosato (Florence, Italy):** „Mechanistic systems biology of metal ions in cells“	**Hermann Schillers (Münster, Germany):** „AFM in Biomedicine: Imaging and Beyond“
18:30 - 19:15	**Claire Wilhelm (Paris, France):** „Intracellular magnetic nanoparticles to image and manipulate the cell“	**Violaine See (Liverpool, UK):** „Single live cell imaging to capture cell to cell heterogeneity and unravel the fine tuning of gene transcription“
19:15 - 19:30	**Delphine Fayol (Paris, France):** „Magnetic cell patterning for tissue engineering applications“	**Louise Ashall (Liverpool, UK):** „Heterogeneity of NF-kappaB oscillations observed in live cells can be explained by transcriptional delay“
19:30	**DINNER**	
20:30		

Wednesday, February 10	Thursday, February 11	Friday, February 12
Jason Crain (Teddington, UK): „Imaging of Host Pathogen Interactions: CARS and two-photon microscopy“	**Philippe Bastiaens (Dortmund, Germany):** „Imaging the spatial organization of signalling networks“	**Nadine Peyrieras (Gif sur Yvette, France):** „In toto imaging for the reconstruction of embryogenesis multiscale dynamics“
Jost Enninga (Paris, France): „Functionalized fluorescence microscopy to correlate the internalization of invasive pathogens with the induced host immune responses“	**Norbert Rolland (Grenoble, France):** „Subcellular and subplastidial proteomics to study intracellular trafficking of proteins“	**Paul Bourgine (Paris, France):** „Reconstructing multi-scale dynamics in morphogenesis of living systems“
Soeren-Oliver Deininger (Bremen, Germany): „MALDI Molecular Imaging for the Classification of HER2 Receptor Status in Breast Cancer Tissues“	**Marius Ueffing (Tübingen, Germany):** „4D approaches to analyse protein networks over space and time“	**Marc Birtwistle (Dublin, Ireland):** „Systems Modeling of Epidermal Growth Factor and Nerve Growth Factor Signaling in PC12 Cells Based on Interaction Proteome Data: New Insights into Transient vs. Sustained ERK **Katrin Büther (Münster, Germany)):** „Bimodal Molecular Imaging of the Endothelin-A-Receptor Expression in Murine Thyroid Cancer Xenografts using Small Animal PET and Optical Imaging“ **Karin Radrich (Neuherberg, Germany):** „Fluorescent Molecular Tomography provides quantitative spatio-temporal resolution of specific molecular processes and diseases“
Andrea Starsichova (Münster, Germany): „Characterization of atherosclerosis and myocardial infarction in a scavenger receptor class B member 1 deficient, hypomorphic apoliprotein E mouse model“	**James Bagnall (Liverpool, UK):** „Intracellular signalling dynamics of Hypoxia-Inducible Factors“	**Maria Athelogou (Munich, Germany):** „Multimodal Image Data Analysis and Quantification“
Julia Lindquist (Turku, Finland): „The intermediate filament nestin as a regulator of myogenic differentiation“	**Denise Bakstad (Liverpool, UK):** „Investigation of the Dynamic Behaviour of p100 and p105 in Single Cells“	**CONCLUSION SSC**
J.M. Liang (Hong Kong): „Ca2+ Response to Acupuncture-induced Acoustic Waves in Cells: More Insights into the Roles of Transient Receptor Potential Channels	**Thomas Viel (Cologne, Germany):** „Dynamic evolution of angiogenesis dependent and independent brain tumor models presenting the tumoral heterogeneity observed in human“	
LUNCH		
		submitted abstracts invited talks
Bob van de Water (Leiden, Netherlands): „Quantitative imaging-based functional genomics screening in cancer research“	**Julio Vera (Rostock, Germany):** „Meta-analysis of strategies for mathematical modelling and data integration in cancer signalling systems biology“	
Xavier Gidrol (Grenoble, France): „Kinome-targeted siRNA screen identifies kinases regulating the diffrentiation/proliferation balance of prostate cancer cells“	**Alexander von Kriegsheim (Glasgow, UK):** „Modelling MAPK crosstalk“	
Wim Vermeulen (Rotterdam, Netherlands): „DNA repair and transcription in living tissue“	**Poster presentation**	
Sandra Zovko (Leiden, Netherlands): „An siRNA-based high-throughput microscopy screen to identify regulators of cell-matrix adhesion dynamics in breast cancer cells“		
DINNER		

content

Silvio Aime, Italy

John Clark, United Kingdom

Andreas H. Jacobs, Germany

Clemens W.G.M. Lowik, The Netherlands

Vasilis Ntziachristos, Germany

Bertrand Tavitian, France

scientific programme committee

thanks for your support

- SYSTEMS BIOLOGY FROM KNOWLEDGE MANAGEMENT TO IMAGES
  Hans Werner Mewes – Munich, Germany
- POLY-B-CYCLDEXTRIN BASED PLATFORM FOR pH MAPPING VIA A RATIOMETRIC $^{19}F/^{1}H$ MRI METHOD
  Francesca Arena – Torino, Italy
- CONSTRUCTION OF PROBABILISTIC DENSITY MAPS FOR MICROPATTERNED CELLS: A COMPUTATIONAL IMAGING APPROACH TO SYSTEMATICALLY STUDY THE GLOBAL CELLULAR ARCHITECTURE OF ENDOMEMBRANES
  Kristine Schauer – Paris, France
- ACTIN AND MAJOR SPERM PROTEIN CYTOSKELETON ASSEMBLY FOR MOTILITY
  Julie Plastino – Paris, France
- MECHANISTIC SYSTEMS BIOLOGY OF METAL IONS IN CELLS
  Antonio Rosato – Florence, Italy
- INTRACELLULAR MAGNETIC NANOPARTICLES TO IMAGE AND MANIPULATE THE CELL
  Claire Wilhelm – Paris, France
- MAGNETIC CELL PATTERNING FOR TISSUE ENGINEERING APPLICATIONS
  Delphine Fayol – Paris, France

day one monday february 8, 2010

Systems Biology from Knowledge Management to Images

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Visual input and associative processing of images in the brain has been a prerequisite for animal evolution. Only very few species are blind. Like moles, they live under special conditions and appear to have lost their viewing ability in an adaptation process. In humans, processing of images in the visual cortex and the ability to store images in memory over long time periods is a fundamental precondition for all kinds of strategies for survival. Even more, the social and cultural achievements governing all kind of human interactions including communication and emotion rely heavily on our visual perception.

Observation of organisms to understand the underlying principles of life from birth to death was the only way to collect information in the early days of research in biology. As always, these visual impressions are projected towards the knowledge that is magically concealed in memory. Images are interpreted by intuitive matching of the new to the known. But what you see is not always what you get and what you see does not allow for the understanding of biological processes. As we know, the molecular level determines the properties of biological systems – but not their fate. For a long time, the mechanisms of causation from genotype to phenotype were buried; Mendel could not have an idea about the molecules involved in heredity, although he could show a clear and causative relation between the phenotype of parents and offspring.

The genome age has inversed our view to the biology: knowing the primary information encoded in the genome has guided the analysis of the molecular makeup of the cell. But like a heap of stones is not a house, a soup of molecules is not a cell (O. Wolkenhauer). This view makes Systems Biology different and it is one of its underlying paradigms: the interaction of molecules restricted by time, space, and condition is the key to understand any biological process. Now, it is time to turn our deductive logic upside down: instead from the mapping of phenomena to the underlying genetics, we must search for the phenotypic changes as the result of the rationale interference of the components.

In my presentation, I will outline the basic paradigms of Systems Biology and their impact on Molecular Imaging and try to develop ideas how Molecular Imaging can drive Systems Biology. I will demonstrate some methods to explore the biological knowledge to interpret data and to guide experiments.

Poly-β-Cycldextrin based Platform for pH mapping via a ratiometric $^{19}F/^{1}H$ MRI method

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Introduction: Mapping pH is an important task in Medical Imaging as changes in pH usually accompany the development of various pathologies including tumors, stroke, infection.

Several paramagnetic metal complexes whose relaxivity is pH-dependent have been reported1. However none of them have been succesfully applied in vivo because in order to have images reporting the pH map it is necessary to transform the observed changes in relaxation rates (R1) in changes of relaxivities (r1). This transformation requires the knowledge of the local concentration of the metal complex. A route to acquire this information may be pursued through the acquisition of the MR image of a heteronuclear signal originated from a molecule that displays the same "in vivo" biodistribution of the paramagnetic complex.

Herein we report a supramolecular construct formed by: i) a Polycyclodextrin substrate that hosts ii) a suitably functionalized pH responsive Gd(III)complex and iii) an analogously functionalized 19F-containing molecule. The binding to the PolyCD substrate is pursued through the introduction of an adamantane group on both Gd and F containing systems. Adamantane is known to have a high binding affinity to β-CD cavities2.

Methods: The Proton Relaxation Enhancement (PRE) method to study the affinity binding of the adamantane functionalized Gd-complex towards Poly-β-CD substrate was performed using Stelar Spinmaster spectrometer at 298 K. The interaction of the 19F-containing reporter to Poly-β-CD has been assessed by measurement of the 1H-NMR chemical shift changes upon formation of the inclusion complex on a Bruker 600 spectrometer. Profiles of water proton relaxivity as a function of pH measured for the supramolecular adduct Poly-CD/19F-reporter/Gd-complex (20mM/5mM/1mM) at 298K and 1 T and 7.1 T on Stelar Spinmaster spectrometer. The proton 1/T1 NMRD profiles were measured over continum of magnetic field strength from 0.00024 to 0.47T on a Stelar field–Cycling relaxometer. Data points from 0.47T to 1.7T were collected on a Stelar Spinmaster spectrometer working at variable field. T1-weighted 1H-MR Images and T1-weighted 19F-MR Images were acquired on a Bruker 300 spectrometer. T1-weighted 1H-MR Images were also acquired on a Aspect spectrometer operating at 1T.

Results: The proof of concept of this approach has been obtained by acquiring the 1H and 19F-MRI images of a phantom consisting of four tubes filled with solutions of Gd/F/PolyCD adduct at different values of concentration and pH. The 1H-MR image does not account for a proportional change in contrast with pH because the observed R1 is dependent on both pH and concentration. Through the acquisition of the 19F-MR image it has been possible to assess the concentration of the adduct in the four tubes thus allowing the R1 →r1 transformation. The method proved to work well with a small (1-2%) error in the pH assessment.

Conclusions: In conclusion, the herein reported results show that a novel, highly accurate method for pH mapping is now available. Finally the Poly-CD/F/Gd adduct can be edowed with targeting properties by hosting in one of the empty β-CD cavities an adamantane functionalized moiety able to provide the system with the proper recognition towards the target of interest.

References

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Construction of probabilistic density maps for micropatterned cells: a computational imaging approach to systematically study the global cellular architecture of endomembranes

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Introduction: Despite a good knowledge of the molecular mechanisms involved in membrane trafficking and membrane homeostasis, the global subcellular architecture of membranous compartments is not known. Taking advantage of adhesive micropatterning technology, which enforces cells to take a certain reproducible shape and impairs migration, we reveal the 3D steady-state organization of several membranous compartments using a computational imaging approach.

Methods: Human retinal pigment epithelial cells were plated on crossbow-shaped-micropatterns (A), fixed, labeled for various intracellular membranes and visualized via fluorophores. Image stacks of fluorescent cells were acquired, deconvolved and segmented to extract the coordinates of labeled endomembranes that were aligned using the micropattern. Using several tens of cells, 2D (B) and 3D (C) probabilistic density maps were calculated to reveal the organization of endomembranes. A statistical test was developed and applied to reveal changes in 3D organization.

Results: Applied to several well-known marker proteins, we revealed the average steady-state organization of early endosomes, multivesicular bodies/lysosomes, endoplasmic reticulum exit sites, the Golgi apparatus and Golgi-derived transport carriers (B,C) in crossbow-shaped cells. The steady-state organization of each tested endomembranous population was well-defined, unique and in some cases depended on the cellular adhesion geometry. Density maps of all endomembrane populations became stable when pooling several tens of cells only (A), and were reproducible in independent experiments, allowing construction of a standardized cell model. Subtle changes in steady-state organization induced by disruption of the cellular cytoskeleton (D) were detected with strong statistical significance requiring only twenty cells.

Conclusions: Combining micropatterning with construction of endomembrane density maps provides a first and powerful tool to systematically study intracellular trafficking determinants. Beyond implications in the field of cellular biology, this interdisciplinary methodology is potentially applicable to the automatic detection of abnormal phenotypes in diverse applications including high-content screening.

Acknowledgement: NikonCenter@IC-CNRS

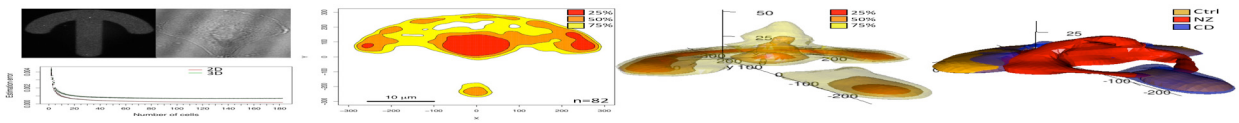

Figure: A B C D

Actin and Major Sperm Protein cytoskeleton assembly for motility

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Crawling behavior of individual motile cells occurs via the assembly of globular cytoskeletal proteins into biopolymer filaments, which pushes out the plasma membrane, coupled with contraction of the cell posterior, which squeezes the cell body forward. In most cells, the cytoskeletal protein actin works with the molecular motor myosin to perform cell locomotion. However the nematode sperm cell displays crawling-type motility in the absence of actin and known molecular motors, and membrane protrusion is produced by the Major Sperm Protein (MSP) cytoskeleton.

We use a combination of in vitro reconstitution of motility and live cell imaging to probe the dynamics of the actin and MSP cytoskeletons during movement. As concerns the actin system, we reproduce cellular actin polymerization in a controlled manner on bead surfaces in order to study how biochemical factors affect movement. We are particularly interested in the Ena/VASP family of proteins as their role in cytoskeleton dynamics is not clear. Overall, our results are consistent with the idea that Ena/VASP has a global effect of detaching actin networks from polymerization activating proteins. Much less is known about the MSP cytoskeleton, but as a first step toward understanding the dynamics of this biopolymer with no sequence or structural homology to actin, we have generated a transgenic nematode strain carrying a fluorescently labeled form of MSP. Using this probe, we measure cytoskeletal flows under different conditions of membrane tension, and observe retrograde flow resembling that of acto-myosin containing cells.

Overall, by using a range of set-ups, from simplified in vitro systems to whole cell imaging, and by comparing the classical actomyosin system to unconventional MSP-based motility, we hope to better understand how cytoskeleton dynamics produce movement and cell shape changes.

Mechanistic systems biology of metal ions in cells

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Metal ions play a large variety of roles in living systems. Depending on their identity, environmental concentration and on the specific chemical species they are involved in, metal ions can be essential for the correct functioning of the cell or highly toxic. Essential metal ions are found as cofactors in enzymes, or as components determining the structural properties of biological macromolecules. To cope with the complexity of interacting with metal ions, living organisms have developed highly sophisticated machineries to control their chemical reactivity as well as their intracellular levels and distribution, through a suitable combined tuning of thermodynamic and kinetic properties. Here we will address the features, molecular mechanisms and reciprocal interactions of some of the key players that contribute to the achievement of the above goals. We will also describe the computational and experimental approaches that can be applied to the investigation of these aspects.

References:

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Intracellular magnetic nanoparticles to image and manipulate the cell

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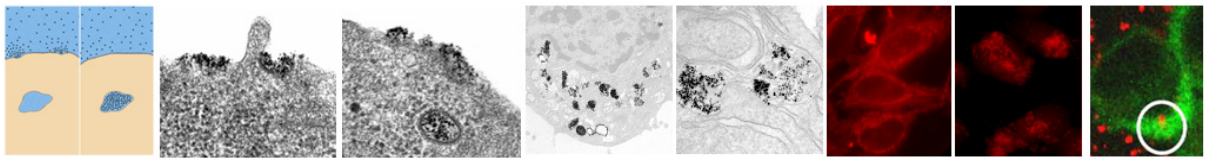
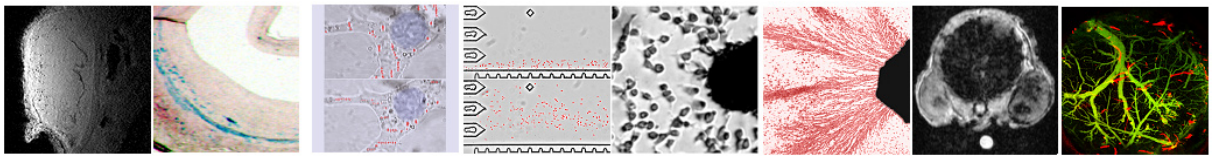


Labeling living cells with magnetic nanoparticles has received increasing interest for the last ten years, mainly because of the emerging method of Magnetic Resonance Imaging (MRI) cell imaging. A few years ago, we proposed the use of anionic iron oxide nanoparticles as efficient agents for cell internalisation. Since this date, we achieved, with these nanoparticles, the magnetic labelling of a large variety of cells, demonstrating that this magnetic cell labelling procedure was ubiquitous and biologically innocuous. Intracellular nanoparticles localisation could be monitored by electron microscopy at the nanoscale, or optical microscopy at the microscale. At the cellular scale, conferring magnetic properties to cells allowed non invasive 3D detection of single cells using MRI. Beyond imaging, the concept of "magnetic cells" opens new possibilities for cell manipulation by non-contact constraints. Magnetic forces at a distance could be used to control the movement of flowing cells (with application in cell sorting), but also to influence the organization and the migration of cells on a substrate (cell magnetotaxis). Inside the cells, the nanoparticles concentrate in pre-existing intracellular membrane-bound vesicles known as endosomes. This renders these compartments magnetic, and allows them to be manipulated within the intracellular environment by applying an external rotating magnetic field in order to explore the local mechanical properties of the cell interior.



Magnetic cell patterning for tissue engineering applications

Fayol D. [1], Gazeau F. [1], Luciani N. [1], Poirier-Quinot M. [2], Letourneur D. [3], Le Visage C. [3], Wilhelm C. [1]

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The purpose of tissue engineering is to induce tissue regeneration or create a functional tissue substitute by combining the use of materials, cells and/or growth factors. In this approach, stem cells are usually seeded into a scaffold which in turn supports their adhesion, growth and differentiation. Controlling the spatial organization of the seeded cells is a key issue in so far as the structure of native tissues plays a central role in their ability to perform their function. So far, cell patterning has been mainly achieved using nanostructuration or chemical modification of the substrate. However, both techniques permanently modify the cell environment. Alternatively, the use of an external force leads to cell patterning while maintaining a non-specific cell environment.

Magnetic cell labeling with anionic maghemite nanoparticles do not affect cell viability or differentiation potential and allows for cell manipulation at a distance with a magnetic field. Here we propose to use the subsequent magnetic force to create 3D cell assemblies inside a 3D scaffold. This 3D magnetic patterning method is developed with a view for two tissue engineering applications. First, endothelial progenitor cells (EPC) can be patterned on the lumen of a tubular gel using a cylindrical magnet. Their subsequent differentiation into endothelial cells may give rise to a vascular prosthesis with an endothelium. Second, the magnetic force can lead to cell confinement, which is a prerequisite for mesenchymal stem cells (MSC) chondrogenesis.

To monitor the tridimensional organization of cells, we used two imaging techniques: confocal fluorescence microscopy and Magnetic Resonance Imaging (MRI), each cell being an MRI contrast agent due to its magnetic label. While the first one offers a better resolution, the second allows for the 3D imaging of the whole scaffold.

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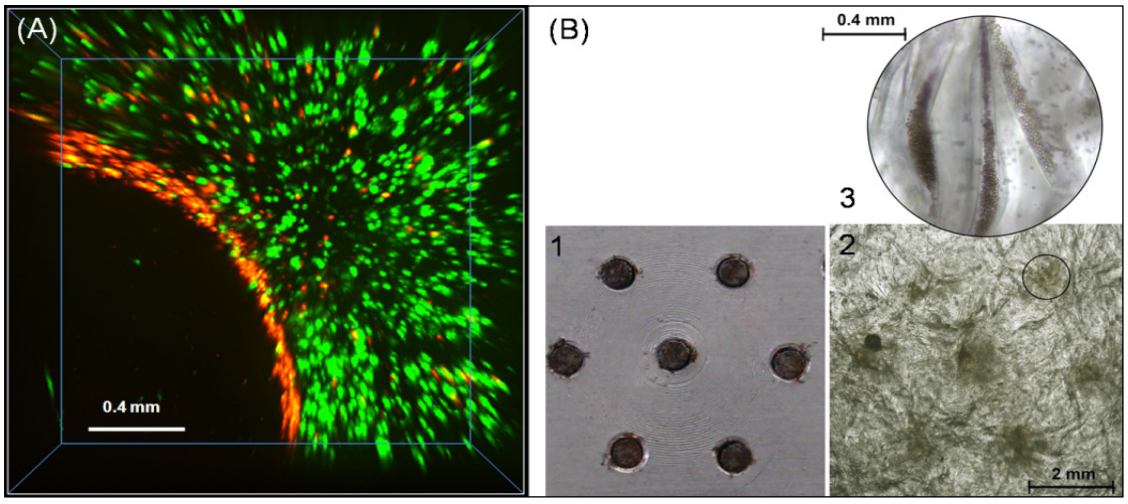

Figure: (A) Using a cylindrical magnet, the lumen of a tubular gel can be patterned with magnetic EPCs (red) while non-magnetic MSC (green) locate in the bulk; (B) A magnetic tip network (1) leads to MSC confinement in lamellar gels (2,3).

- A SYSTEMS MICROSCOPY ANALYSIS OF CELL ADHESION AND MIGRATION – APPLICATIONS TO NOVE ADHESION BIOLOGY
  John Lock – Huddinge, Sweden
- *in vivo* IMAGING OF CELL MIGRATION IN ZEBRAFISH
  Elena Kardash – Münster, Germany
- IMAGING OF LIVING ZEBRAFISH EMBRYOS IN A MICROFLUIDICS LAB-ON-CHIP
  Michael K. Richardson – Leiden, The Netherlands
- MULTIPURPOSE BIOCHIP: A NOVEL 4D IMAGE AND DATA ACQUISITION TECHNOLOGY FOR DRUG DISCOVERY
  Eric Marjin Wielhouwer – Leiden, The Netherlands
- SYSTEMATIC ANALYSIS OF GENE EXPRESSION PATTERNS IN DROSOPHILA EMBRYONIC DEVELOPMENT
  Pavel Tomancak – Dresden, Germany
- VISUALIZATION OF ZEBRAFISH CORNEA DEVELOPMENT
  Masanari Takamiya – Karlsruhe, Germany
- IMAGE ANALYSIS FOR AUTOMATED LIVE-CELL IMAGING
  Dimitris Kalamatianos – Maynooth, Ireland
- AFM IN BIOMEDICINE: IMAGING AND BEYOND
  Hermann Schillers – Münster, Germany
- SINGLE LIVE CELL IMAGING TO CAPTURE CELL TO CELL HETEROGENEITY AND UNRAVEL THE FINE TUNING OF GENE TRANSCRIPTION
  Violaine See – Liverpool, UK
- HETEROGENEITY OF NF-kappaB OSCILLATIONS OBSERVED IN LIVE CELLS CAN BE EXPLAINED BY TRANSCRIPTIONAL DELAY
  Louise Ashall – Liverpool, UK

day two tuesday february, 9 2010

A Systems Microscopy Analysis of Cell Adhesion and Migration – Applications to Nove Adhesion Biology

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Introduction: Cell-extracellular matrix (ECM) adhesion and migration are emergent processes arising from a diverse network of mechanical and signaling interactions that are scaled from the molecular to the cellular level, and are distributed in spatiotemporally resolved and asymmetric fashion. To understand the physical mechanics and information fluxes that compose and control this intricate system, we have developed an automated fluorescence imaging and image analysis pipeline that permits the quantitative integration of over 100 parameters describing the distribution, morphology and dynamics of features from the molecular to the cellular level. This approach has recently been applied to characterise a novel class of cell-ECM adhesion complexes revealing unique properties the underlie a potentially crucial role in cell mitosis.

Methods: We apply high resolution, automated, live and fixed cell fluorescence imaging techniques followed by automated image analysis and quantitative data extraction using custom developed software, to create a rich and highly dimensioned characterisation of molecular- to cellular-level properties of cells during adhesion, migration and cell division. This pipeline is supplemented by an ever expanding raft of mathematical and statistical techniques which allow the interrogation of numerous properties, from the distributions of individual features to the shifting network relationships between each and every variable measured.

Results: Here I will define some of the basic capabilities of this approach, and then demonstrate how these tools have been applied to identify and characterise a novel class of cell-ECM adhesion complexes. Notably, these complexes appear to re-define the dogma of integrin adhesion biology in terms of integrin dependence on factors such as Talin and Actin for activation and complex stabilisation. Further, I will present preliminary data revealing a putative role for these adhesions in the transmission of spatial memory from parent to daughter cell generations during cell division.

Conclusions: In order to enhance our understanding of how cellular machineries (such as adhesions and actin) are organised and engaged in the adhesion/migration process, it is crucial to create a temporally resolved, quantitative, comprehensive and integrated analysis of key properties ranging from the molecular to the cellular level. By repeating such analyses under a wide range of perturbed conditions, where specific pathways and molecules are targeted, we believe it will be possible to build a substantially improved understanding of the signals and mechanisms controlling cellular adhesion and migration.

In vivo imaging of cell migration in zebrafish

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Germ cell migration in zebrafish serves as a model system for studying directed cell migration in vivo. Germ cells in zebrafish are specified early in development in multiple locations and are guided towards their target by the chemokine SDF-1a, which activates its receptor CXCR4b that is expressed in the germ cells [1]. The actual motility of germ cells and the associated cell shape changes are characterized by the formation of blebs that are generated preferentially at the leading edge of the cell in response to local elevation of calcium [2]. The position where calcium is elevated can be controlled by CXCR4b, thereby allowing SDF-1a to bias the direction of the migration.

To understand the mechanics of blebs-assisted cellular movement in a three-dimensional environment, we studied the role of Rac and RhoA in controlling actin cytoskeleton dynamics in migrating germ cells and analyzed the role of cell adhesion in this process. Using deregulated versions of Rac1 and RhoA we could demonstrate that the function of these proteins is critical for controlling proper germ cell shape and migration. Using FRET biosensors we aimed to determine the site within the cell where these Rho GTPases function. We first optimized FRET imaging for Rac and RhoA activity using Raichu probes [3, 4] introducing several modifications to the original constructs. Additionally, we used mutants to distinguish between the active and the inactive forms of the sensor within the cell, thus validating the measurements of the activation state of Rac and RhoA in a wild type situation. We could demonstrate that Rac function at the front of the cell induces the formation of actin-rich structures, while RhoA action promotes actin retrograde flow at the same location. We then explored the role of cell-cell adhesion in germ cell migration by interfering with the function of E-cadherin in germ cells and could demonstrate that this molecule is essential for germ cell migration in vivo.

Together, our findings suggest that actin retrograde flow at the front of germ cells is essential for the generation of E-cadherin-mediated traction forces that are crucial for cell motility *in vivo* (Figure 1).

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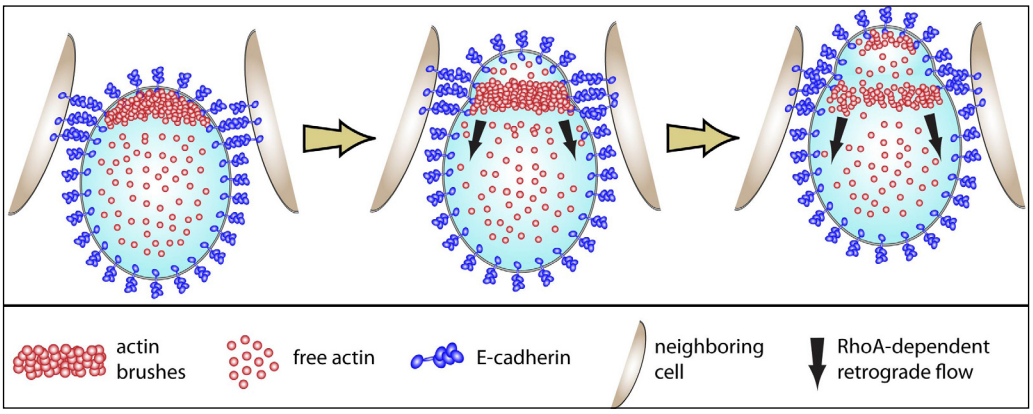

Figure 1: A model for cooperation between blebbing, actin retrograde flow and e-cadherin-mediated traction force in germ cell motility.

A WHOLE EMBRYO LAB-ON-A-CHIP FOR ZEBRAFISH

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We have designed, patented and prototyped a glass microfluidics chip for culturing zebrafish embryos. The Chip is of trilaminar construction and contains 32 wells in which zebrafish embryos are fed constantly with buffer or egg water. The wells are arranged in parallel to prevent cross-contamination. Using the chip, we can culture zebrafish embryos for up to 5 days after fertilization, by which time they have hatched and have most of the mayor organ systems present and functional. This chip is optically transparent and is compatible with fluorescent microscopy. In particular, imaging is performed from top and/or bottom of the Biochip using upright, inverted or confocal microscopy. Compared to standard 96 well plate cultures, our chip offers extreme low culture volume, thereby saving on reagents. Also the biochip has the ability to remove heat generated by confocal imaging though temperature channels located aside of each well. It also provides dynamic replacement of buffer rather than the very stressful static replacement - or no replacement - of buffer in standard assays. In view of the growing demand for alternatives to mice in drug and cosmetics testing, we hope this chip will find application in industry and academia.

Multipurpose BioChip: A novel 4D image and data acquisition technology for drug discovery

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Whole animal screening is labour and cost intensive and consumes the patent life of lead compounds (potential future medicines). Therefore, pharmaceutical companies, the biotech sector and academia used immortalized cells from skin, muscle, liver, etc. to perform low cost pre-screening of compound libraries. A major limitation of cell lines is that they cannot model organismal-level disorders (e.g. headaches or toxic side effects of secondarily metabolites). Our BioChip is a microfluidic device that enables whole animal screens to be performed at the high throughput and high content level. By reconstructing 3D images at different developmental stages, we can carry out 4D screening. This is an advance on current "low-throughput" technologies such as multiwell plates. We present here data on the culture and imaging of Tribolium and zebrafish. We tested our BioChip, made of glass, using whole organisms (Tribolium beetle and zebrafish embryos). Embryos were cultivated in flow-through conditions of air or buffer, respectively. The zebrafish grew normally for 5 days. Administration of 10 percent ethanol for one hour at 26 somite and prim-6 stage results in morphological abnormalities whereas controls show no side effects using 32well BioChips and 96well microtiter plates. Also, we show Fli gene expression pattern in zebrafish. Finally, we show testing of gaseous or evaporated compounds on insects belong also to the possibilities.

Systematic analysis of gene expression patterns in Drosophila embryonic development

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Introduction: To understand a developmental system means to understand how the information contained in the genome transforms into coordinated cellular behaviors that collectively represent development. It can be argued that the most direct manifestation of the genomic program is the tissue specific regulation of gene expression. Therefore, by describing the gene expression patterns in the context of cellular anatomy of the developing system, we take the necessary, first step towards understanding the information transfer from genome sequences to developmental processes.

Methods and Results: In the past, we systematically documented gene expression patterns during Drosophila embryogenesis by assembling large collection of annotated images of RNA in situ hybridizations [1-2]. Recently, we developed molecular and imaging techniques that enable recording of gene expression patterns in entire living embryos. In order to visualize gene expression patterns in live embryos, we constructed two complementary genomic fosmid libraries (FlyFos) for Drosophila melanogaster and Drosophila pseudoobscura that permit seamless modification of large genomic clones by high-throughput recombineering and direct transgenesis [3]. The fosmid transgenes rescue mutant phenotypes, recapitulate endogenous gene expression patterns and in some cases allow imaging of gene products in living animals. The D. pseudoobscura transgenes rescue RNAi phenotypes when introduced into the D. melanogaster genome, providing a convenient control for the specificity of the knockdown. These libraries will, in combination with recombineering technology, enable systematic analysis and manipulation of gene activity across species. We plan to leverage the astonishing efficiency of our toolkit to establish a genome-wide resource of tagged fosmid transgenes [4].

To image the activity of the transgenes we chose Selective Plane Illumination Microscopy (SPIM) that allows in toto imaging of large specimens, such as Drosophila embryos, by acquiring image stacks from multiple angles. We developed an algorithm for registration of multi-angle microscopy acquisitions using fluorescent beads in rigid mounting medium as fiduciary markers. So far we obtained unprecedented 4D SPIM recordings of embryos expressing His-YFP in all cells throughout embryonic development. We show that the approach can be used for imaging and registration of multi-angle acquisition in any imaging modality. We present our image analysis approaches in the form or usable software solutions maintained and extended under the Fiji (Fiji Is Just ImageJ) Open Source image processing platform [5].

Conclusions: Together, these two sets of tools will enable quantitative, digital description of the dynamics of gene expression in developmental systems on the cellular level and form the observational foundation for developmental systems biology of Drosophila embryogenesis.

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Visualization of zebrafish cornea development

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Introduction: The cornea plays an important role for a normal vision by refracting the light (especially for terrestrial animals) and provides a transparent window, whose dysfunction explains one-third of 45 million cases of blindness in 2004. Recent DNA microarray studies in higher vertebrates revealed sets of genes expressed in the cornea from postnatal to adult stages and gave hints to understand its maintenance machinery: extracellular matrix components, ion transport and so on. However, developmental aspects of how this machinery becomes established during early development is not yet understood simply due to the lack of proper model organism.

Methods: We used zebrafish as a model organism to answer this question. Two different approaches were chosen: in vivo imaging and DNA microarrays.

Results: To visualize cornea development, we focused on the neural crest cells, whose contribution to the cornea formation is evolutionally conserved. We established a transgenic system to visualize the undifferentiated state of the neural crests by combining sox10 promoter whose activity is limited to multi-potent neural crests and a photo-convertible fluorescent protein (sox10::eosFP transgenic line); photo-conversion prior to the time lapse imaging allows one to discriminate earlier reporter products from the later. We found neural crest cells form multiple temporal clusters in the developing pharyngeal arches, before they reach and differentiate into various structures. We are currently developing an image-processing protocol to evaluate the effects of genetic manipulation or chemical treatments on the neural crest cell migration, to gain insights into the responsible pathways. Next, to identify genes involved in the maintenance of the zebrafish cornea, we analysed gene expression profile of the adult cornea using the skin as a reference tissue, which is initially continuous with the cornea during embryonic stages and shares high anatomical similarities. Microarray analysis showed ~165 cornea genes that are overrepresented in the adult cornea in comparison to the skin ($P<0.01$). We analyzed endogenous spatial distribution of the cornea genes in the embryonic and adult tissues by in situ hybridisation, in combination with bioinformatic analysis on zebrafish gene expression database. We found that genes overrepresented in the adult cornea are initially shared with and co-expressed in the skin.

Conclusions: We propose a hypothesis that cornea is a default state of the two surface structures (cornea or skin) in zebrafish. A role of neural crest cells in this differentiation process will be discussed.

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Image analysis for automated live-cell imaging

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Live cell imaging and fluorescent microscopy have gained increasing interest during the last decades as they allow the study of cellular physiology and signal transduction in response to stimuli in-vivo and in real time [1]. However, they require significant investment in time and effort in data handling of multivariate time series. Moreover, cellular events during measurement often occur spontaneously after a long lag time and then proceed rapidly upon initiation [2]. To overcome these drawbacks, we have developed ALISSA – an automated live-cell imaging system for signal transduction analyses [3]. ALISSA allows the detection of intra-cellular events indicated by changes of fluorescence intensities and subsequently exercises control over confocal microscopes to change modalities of image acquisition such as sampling time or additional laser resources. This results in a significant decrease of manual workload as well as reduction of phototoxicity and photo-bleaching.

In order to achieve this, it was necessary to develop and implement advanced image processing techniques for the segmentation and tracking of cells. To perform this in real time and online during an experiment, these algorithms should require minimum computation cost and time. Several classical segmentation algorithms were adapted and implemented in the ALISSA system. These comprise local maxima and circular seeded watershed [4-5], and modified constrained erosion-dilation [6] algorithms. To track cells over time, we first find an accurate estimate of the new cell shape and position using a condensation filter [7] and then update this estimate using a robust expression of active contours to refine the shape, based on vector field convolution [8]. From the tracked cells, the image analysis tool of ALISSA extracts fluorescence readouts for each channel and cell which are used for event detection and are stored in a database for further offline analysis.

ALISSA's benefits range from automated segmentation and tracking to adaptive change of image modalities and online evaluation which we have demonstrated by way of a reference application (apoptosis). By switching on the high-energy lasers only for short time periods and within limited regions of interest, the total amount of laser radiation energy absorbed by the sample during experimentation is significantly lower when using ALISSA compared to conventional time-lapse imaging.

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AFM in Biomedicine: Imaging and Beyond

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Advances in biology have been closely related with the development of microscopy techniques. The atomic force microscope (AFM) is a tactile instrument which provides three-dimensional images of a sample by scanning a sharp probing tip over the sample surface. The advantage of AFM, compared to other high resolution imaging techniques, is to operate under physiological conditions. This enables investigation of the dynamical behavior of living cells and subcellular components down to single protein level. Although AFM imaging provides outstanding structural information of biological samples further developments of this technique gain insight into biophysical and chemical characteristics of cells and cell membranes.

Tip modification improves the ability of the AFM to visualize the protein composition of a sample while mapping its topographic structure. Identification of a specific protein is achieved using an antibody covalently bound to the scanning AFM tip. This approach provides both sub-molecular spatial resolution and direct protein recognition via antibody-antigen interaction. We used this so called TREC imaging (Topography and RECcognition) to quantify the protein cystic fibrosis transmembrane conductance regulator (CFTR) in human erythrocyte cell membranes of healthy donors and cystic fibrosis patients. Cystic fibrosis is a lethal disease characterized by impaired electrolyte transport and fluid secretion of several epithelia, including the sweat duct, exocrine pancreas, and the pulmonary airways. It was shown that the membrane distribution of CFTR in cystic fibrosis patients is tissue-specific and exhibits variation of expression from null to apparently normal amounts. With TREC we could show that the number of CFTR molecules is reduced by 70% in erythrocytes of cystic fibrosis patients.

Beyond imaging AFM is used to apply force to living cells in order to obtain biomechanical information. Nearly all cells in our body are continuously stretched and squeezed, e.g. lung tissue and vascular endothelium. Cells elasticity is mainly determined by the cytoskeleton, a highly regulated and very dynamical system. The principle of elasticity measurement is to physically indent a cell with an AFM probe, to measure the applied force and to process this force-indentation data using an appropriate model. We used this technique to monitor biomechanical dynamics of bronchial epithelium under various conditions for a functional characterization of the cytoskeleton in living cells.

Cells convert physical forces into biochemical signals, the mechanotransduction, which clearly depends on cells compliance. We investigated the compliance /stiffness of vascular endothelium subjected to different extracellular potassium / sodium concentrations and under variations of sodium channel (ENaC) activity and measured the nitric oxide release. We further developed a method which allows simultaneously measurement of mechanical stiffness and electrical membrane potential by a combination of AFM and epifluorescence.

Our results show that disturbance of sodium/potassium homeostasis alters endothelial cell stiffness and that endothelial deformability controls nitric oxide release. This salt sensitivity may serve as a physiological feedback mechanism to regulate local blood flow and thus influence systemic blood pressure. Therefore we assume that alterations of endothelial cell stiffness may contribute to the development of hypertension.

AFM-based techniques are versatile tools to study interrelations of biomechanical forces and cellular processes. Understanding these correlations should help to elucidate function and regulation of more complex systems like tissues and finally organisms.

Single live cell imaging to capture cell to cell heterogeneity and unravel the fine tuning of gene transcription

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At a given time-point, cells in a population are heterogeneous in their functions and fate and it is therefore vital to develop and apply methods that allow the measurement of dynamic molecular processes in single cells. We have previously shown, using single cell imaging, the critical role of nucleo-cytoplasmic localisation oscillations of the NF-κB transcription factor to control downstream pattern of gene transcription (Nelson *et al*, Science 2004; Ashall *et al*, Science 2009). In most cases, oscillations had previously been masked in population level studies by cellular heterogeneity. The level of cell to cell heterogeneity is due in part to un-synchronised dynamics in single cells, which may involve oscillatory processes and/or cross-talk with extrinsic signals. We have also observed cell to cell heterogeneity in other signalling systems such as in cellular response to low oxygen environment (hypoxia). In both inflammatory and hypoxic signalling systems one source of heterogeneity is due to the presence of extrinsic dynamic processes that are functionally coupled and that are occurring over different time scales. We identified the cell cycle as one source of variability as cells must coordinate and prioritise their response to the environment depending on their cell cycle status. We apply mathematical modelling using the quantitative data generated by imaging experiments to predict the role of the negative feedback, to unravel new network motifs and to characterise the cross-talk between the signalling systems.

Heterogeneity of NF-kappaB oscillations observed in live cells can be explained by transcriptional delay

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Introduction: More than 20 years of research has now been dedicated to the NF-κB signalling pathway due to its importance in the critical control of cellular responses. NF-κB is activated by a large range of biological factors and environmental conditions, which in turn regulates the expression of genes involved in the immune system, inflammatory responses, cell growth, cell survival and apoptosis. NF-κB is a dynamic transcription factor that is regulated by inhibitors known as IκB's that include –α, -β and -ε isoforms. Transcription of the IκB's is controlled by NF-κB itself, creating a delayed negative feedback loop that primes the system for oscillations. Here we investigate in detail, the interplay between the two key IκB isoforms; IκBα and IκBε, and their role in the modulation of the NF-κB oscillations both at the single cell and population level.

Methods: ive cell time lapse imaging was used to observe the intracellular dynamics of NF-κB translocations. In order to determine the effects of IκBε ablation on the p65 dynamics following Tumour Necrosis Factor-alpha (TNFα) stimulation, cells were transiently transfected with plasmid to express p65-dsRedxp along with either control or IκBε siRNA. In addition, an existing mathematical model of the NF-κB pathway was extended to include stochastic regulation of gene activity in order to analyse the NF-κB oscillations in wild-type and IκBε-knock down cells.

Results: Previously it was shown that in mouse embryonic fibroblasts, TNFα-induced NF-κB-mediated transcription of IκBε was delayed by 45 min compared to that of IκBα (1). A similar delay in the transcription of IκBε was observed in both SK-N-AS and HeLa cells. Further investigation showed that the timing of p65 binding to the IκBα and IκBε promoters was similar yet IκBε transcription was delayed compared to that of IκBα. A likely reason for this was the delayed recruitment of RNA polymerase II to the IκBε promoter. The role of IκBε in regulating p65 dynamics was examined in detail. The mathematical model predicted that stochastic variation from delayed IκBε feedback should generate enhanced cell to cell heterogeneity in wild-type cells compared to IκBε knock-out cells and that the 45 minute delay induced maximum heterogeneity. Experimental siRNA knock-down of IκBε feedback in SK-N-AS cells had no effect on oscillation amplitude and peak to peak timings compared to control cells, contradicting the previous prediction that IκBε feedback might dampen oscillations in wild-type cells (1).

Conclusions: This combined experimental and computational study predicted how negative feedback loops regulate the dynamics of the system. We found that in both wild-type and IκBε knock-down single cells, the NF-κB cytoplasmic-nuclear oscillations were of similar amplitude and timing. The stochastic mathematical model predicts that the delay in the transcription of IκBε increases the level of stochasticity in the system and explains the source of cellular heterogeneity showing that oscillations are an important characteristic of the NF-κB response to TNFα (2).

Acknowledgement: This work has been funded by the MRC and BBSRC.

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- IMAGING OF HOST-PATHOGEN INTERACTIONS: CARS AND TWO-PHOTON FLUORESCENCE MICROSOPY
  Jason Crain – Teddington, UK
- FUNCTIONALIZED FLUORESCENCE MICROSCOPY TO CORRELATE THE INTERNALIZATION OF INVASIVE PATHOGENS WITH THE INDUCED HOST IMMUNE RESPONSES
  Jost Enninga – Paris, France
- MALDI MOLECULAR IMAGING FOR THE CLASSIFICATION OF HER2 RECEPTOR STATUS IN BREAST CANCER TISSUES
  Sören-Oliver Deininger – Bremen, Germany
- CHARACTERIZATION OF ATHEROSCLEROSIS AND MYOCARDIAL INFARCTION IN A SCAVENGER RECEPTOR CLASS B MEMBER 1 DEFICIENT, HYPOMORPHIC APOLIPROTEIN E MOUSE MODEL
  Andrea Starsichova – Münster, Germany
- THE INTERMEDIATE FILAMENT NESTIN AS A REGULATOR OF MYOGENIC DIFFERENTIATION
  Julia Lindquist – Turku, Finnland
- CA2+ RESPONSE TO ACUPUNCTURE-INDUCED ACOUSTIC WAVES IN CELLS: MORE INSIGHTS INTO THE ROLES OF TRANSIENT RECEPTOR POTENTIAL CHANNELS
  Jieming Liang – Hang Kong
- QUANTITATIVE IMAGING-BASED FUNCTIONAL GENOMICS SCREENING IN CANCER RESEARCH
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day three wednesday february 10, 2010

Imaging of host-pathogen interactions: CARS and two-photon fluorescence microsopy

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Biomedical research is placing increasing demands on cell imaging techniques for improved resolution, sensitivity and chemical. Intracellular imaging is emerging as a particularly important tool in the investigation of host-pathogen interactions. Advances in this area are particularly sought to understand the effect of viral infection processes on the host cell and its metabolic functions including those cases where host cell lipid metabolism is modulated as a result of infection. In this talk we describe the the use of coherent anti-Stokes Raman scattering (CARS) and two-photon fluorescence microscopies to image live fibroblast cells infected by cytomegalovirus. CARS emission in the C-H stretch region of the spectrum is used to image the host cell membrane, lipid droplets and morphology of the nucleus. Morphological changes in the cell nuclei are monitored during infection. Also, cells show accumulation of lipid droplets at the nuclear periphery. A genetically modified viral strain expressing green fluorescent protein also enables two-photon imaging of the same cells to reveal the location, nature and extent of viral protein expression. This study demonstrates the utility of combining CARS and two-photon imaging modalities to establish correlations between viral protein expression and perturbations to the host cell.

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Functionalized fluorescence microscopy to correlate the internalization of invasive pathogens with the induced host immune responses

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Introduction: One strategy employed by a number of pathogens is the secretion of bacterial effector proteins into host cells via sophisticated secretion machineries. The type III secretion system (T3SS) is one of such machines resembling a molecular syringe. The entero-invasive pathogen Shigella uses this molecular needle to inject about 25 bacterial effectors into the host cells leading to the uptake of the bacterium by typically non-phagocytic host cells, and furthermore the injected undermine the host immune system. The next step of Shigella invasion is the rupture of the endocytic vacuole after uptake. Generally, cellular uptake of bacterial pathogens leads to (i) blocking the progression of the bacteria-containing endosomal vacuoles (e.g. M. tuberculosis), (ii) altering the composition of the endosomal vacuoles, or (iii) escaping the endocytic vacuole via rupture. Studying these events has given novel insight into the mode of function of the participating constituents of the vesicular trafficking machine. However, the overall sequence and hierarchy of the involved steps are still poorly understood due to the limited number of approaches that allow dynamic and functional investigations on the single cell level.

Methods: We have established novel fluorescence microscopy based approaches that give precise information on the bacterial effector secretion, on the intracellular localization of bacterial pathogens, and on the induced host immune responses. With our novel assays, we are able to study how bacterial factors communicate with host factors to shape the localization of the pathogens during cellular uptake.

Results: Using our novel fluorescence microscopic approaches, we found that the Shigella T3SS is induced instantly upon host cellular contact, and that a number of bacterial effectors are injected within 4 to 8 minutes into the targeted cells. Subsequently, vacuolar rupture occurs in less than 10 minutes after the bacterial internalization. Finally, using our novl approaches to monitor gene expression in single living cells, we are able to deduce which signaling cascades are induced before vacuolar rupture and after vacuolar rupture.

Conclusions: Overall, we are able to combine our novel approaches with assays that track the localization of invading bacteria and their physiological behavior. This leads to integrative information on the invasion strategies employed by the pathogens and on the induced host responses.

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MALDI Molecular Imaging for the Classification of HER2 Receptor Status in Breast Cancer Tissues

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MALDI molecular imaging is a technique that allows the direct and untargeted acquisition of molecular images. The detected compounds can be lipids, metabolites, peptides or proteins. Every dataset can contain the distribution of hundreds of compounds at once. The molecular images can be unambiguously correlated with the full histological information. It is thus possible to get a direct molecular insight in a system as complex as tissue. We have applied the technique here to investigate molecular differences in human breast cancer.

Clinical laboratory testing for HER2 status in newly diagnosed, primary breast cancer tissues is critically important for therapeutic decision making. Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a powerful tool for investigating proteins through the direct and morphology-driven analysis of tissue sections. Unlike immunohistochemistry (IHC), MALDI-IMS enables the acquisition of complex protein expression profiles without any labeling. We hypothesized that MALDI-IMS may determine HER2 status directly from breast cancer tissues.

Breast cancer tissues (n=48) predefined for HER2 status by IHC and fluorescence-in-situ-hybridization (FISH) were subjected to MALDI-IMS and protein profiles were obtained through direct analysis of tissue sections. Protein identification was performed by tissue micro-extraction and fractionation followed by top-down tandem mass spectrometry on a spherical ion trap with ETD. A discovery and an independent validation set were used to predict HER2 status by applying proteomic classification algorithms.

We found that specific protein/peptide expression changes strongly correlated with the HER2 over expression (m/z 4740, 8404, 8419, 8455, 8570, 8607, 8626). Among these, we identified m/z 8404 as Cysteine-rich intestinal protein 1 (CRIP1). Of particular note, the proteomic signature was able to accurately define HER2-positive from HER2-negative tissues achieving high values for sensitivity of 83%, for specificity of 92% and an overall accuracy of 89% (95% CI: 65% to 99%).

Our results underscore the potential of MALDI-IMS proteomic algorithms for morphology-driven tissue diagnostics such as HER2 testing and show that MALDI-IMS can reveal biologically significant molecular details from tissues which are not limited to traditional high-abundance proteins. CRIP1 is a cytosolic protein that is potentially useful for serum based diagnostics of HER2 if tissue leakage can be demonstrated.

Characterization of atherosclerosis and myocardial infarction in a scavenger receptor class B member 1 deficient, hypomorphic apoliprotein E mouse model

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Introduction: Atherosclerosis is the most common pathologic process that leads to serious cardiovascular events such as myocardial infarction. However, most of the widely recognized and used animal models of atherosclerosis are not associated with clinical features of the disease resulting for example in coronary occlusion and myocardial infarction. Recently, a new transgenic mouse model has been reported being deficient in Scavenger Receptor class B member 1 (SR-B1-/-) and expressing reduced levels of Apoliprotein E - R61 (ApoE-R61hypo/hypo). Under high fat/high cholesterol chow diet these mice develop occlusive coronary atherosclerosis, myocardial infarction and cardiac dysfunction (1). The aim of the present study was to further characterize this model using molecular biological and imaging methods.

Methods: The individual development of myocardial infarctions in SR-B1-/-/ApoE-R61hypo/hypo mice, either set under high fat/high cholesterol (HFC) diet or continuing with normal chow diet (control), was investigated by serial F-18 -FDG-PET measurements. Histology was used to demonstrate plaque lesions and myocardial fibrosis. Immunohistochemistry was performed to determine cell death, inflammation and myocardial remodelling. In addition, blood samples were collected before and 3 weeks after onset of HFC-diet for blood count analysis.

Results: All experimental mice were fed a normal chow diet before starting the experiments. In contrast to the normal chow diet group, in which no mortality was observed, all animals of the HFC diet group died within four weeks after onset of the diet. Histological analysis of the explanted hearts of SR-B1-/-/ApoE-R61hypo/hypo mice under HFC diet revealed massive plaque development in the coronary arteries, coronary occlusion and extensive fibrosis in the myocardium. TUNEL and caspase-3 immunohistochemistry demonstrated cell death, MAC-3 and MRP-14 staining confirmed leukocytes infiltration and inflammation, MMP-9 staining indicated myocardial remodelling. A significant increase in the number of leukocytes in mice fed HFC diet evidenced for severe systemic inflammatory processes. PET scans performed at various time points demonstrated myocardial infarctions, which only occured in SR-B1-/-/ApoE-R61hypo/hypo mice under HFC diet.

Conclusions: Taken together, our data demonstrate that SR-B1-/-/ApoE-R61hypo/hypo mouse model represents a valid animal model for biological and imaging studies of processes associated with all levels in the pathogenesis of atherosclerosis, from plaque formation to coronary occlusion and myocardial infarction.

Acknowledgement: This work was partly supported by grants from the Deutsche Forschungsgemeinschaft (DFG), Sonderforschungsbereich 656 „Cardiovascular Molecular Imaging“, Münster, Germany (project Z2).

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The intermediate filament nestin as a regulator of myogenic differentiation

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Inroduction: Nestin is an intermediate filament protein generally considered to be a stem cell marker as it is expressed in certain immature cells and some cancers, but accumulating data suggest wide-ranging functions for this protein. Besides providing structural support, nestin also regulates a variety of signaling processes reaching from cell migration to regulation of molecular distribution. During differentiation and regeneration of muscle tissue, nestin is transiently expressed and it was earlier shown to provide a signaling scaffold for cyclin dependent kinase 5 (Cdk5). Intriguingly, nestin regulates the apoptotic activity of Cdk5. Thus, the potential of nestin to regulate several signaling processes is obvious, but its function is still not completely understood. Cdk5, in turn, has been reported to regulate both apoptosis and myogenic differentiation. The activity of Cdk5 is controlled in a strict spatiotemporal manner by the activator p35 and its cleavage product p25, the generation of which is associated with re-localization and hyperactivity of the kinase. We wanted to examine the role of nestin in myoblasts and understand how muscle differentiation is regulated. We were able to show that nestin indeed affects the pace of muscle differentiation presumably through interactions with the Cdk5/p35-signaling complex.

Methods: We used both C2C12 myoblasts and primary myoblasts to investigate muscle differentiation. The effect of nestin on differentiation was illustrated by Western blotting and imaging of differentiating cells at appropriate time points. Cells were transfected using JetPEI- or Lipofectamine reagents.

Results: Nestin regulates myogenic differentiation as overexpression of nestin markedly delayed the differentiation of C2C12 myoblasts observed by Western blotting and immunostaining of myogenic markers. The opposite effect was observed by downregulating nestin through RNA interference. We also showed that nestin affects the early onset of differentiation through influencing the expression of cyclin inhibitors p21 and p27, crucial for the commitment of myoblasts for differentiation. In addition, downregulation of nestin accelerated differentiation of primary myoblasts that were grown from satellite cells isolated from limbs of neonatal mice. The proliferation of C2C12 myoblasts was in turn not affected pointing out that nestin does not affect the cell cycle until the onset of differentiation. Interestingly, downregulation of nestin during differentiation also induced formation of p25 which could denote that nestin would regulate the activity of Cdk5 among other possible targets. Cdk5 seems in turn to be able to regulate its own intermediate filament scaffold.

Conclusions: Our findings suggest that nestin is required for appropriate regulation of early myogenic differentiation. We are interested to investigate further the interplay between nestin and Cdk5 during myogenesis and in other cell systems, using imaging based techniques. The advantages of exploring nestin and Cdk5 are apparent due to their proposed functions in differentiation models, in determining cell fate and regulating cancer cell properties. An important approach will utilize the Leica TCS SP5 Matrix confocal microscope, a powerful tool for high-content live imaging at the Cell Imaging Core at Turku Centre for Biotechnology.

Ca2+ Response to Acupuncture-induced Acoustic Waves in Cells: More Insights into the Roles of Transient Receptor Potential Channels (I)

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Introduction:Acupuncture typically involves manual needle manipulation after needle insertion, which consists of rapidly rotating (back-and-forth) or pistoning (up-and-down motion) of the needle. In attempt to shed light on acupuncture mechanism, we have visualized acoustic wave produced by the mechanical movement of the acupuncture needle in human subjects[1]. The same acoustic wave evoked robust Ca2+ transients in fibroblasts[2], endothelial cells, cardiac myocytes, neurons[3], and in mouse skeletal muscle in vivo[4], providing the first direct link of acupuncture to intracellular signaling events. In search of its molecular basis, this study tries to pinpoint the specific Ca2+ channels responsible for transducing the acoustic wave signals into Ca2+ excitation.

Methods: Murine microvascular endothelial cells (H5V) were used as a cell model. Stealth Select RNAi siRNA duplexes were synthesized to inhibit gene expression. Validation of RNAi knockout was performed by qRT-PCR and western blotting. A needle shear wave driver described before[1-4] was used to generate propagating waves during confocal Ca2+ imaging.

Results: In Fig.1a, acoustic wave induced Ca2+ excitation in H5V, but fail to elicit Ca2+ transient in cells treated with SACC blockers, 100μM Gd3+ or 200μM streptomycin. The results show that endothelial cells respond to acoustic waves by Ca2+ activation through stretch-activated cation channels (SACCs).

SACCs belong to the transient receptor potential (TRP) ion channels superfamily. In mammals, about eight TRP channels in four subfamilies are thought to be sensitive to mechanical forces while being Ca2+-permeable. Of these, there were relatively high Trpc1, Trpv4, Trpm7 and Trpp2 mRNA levels in H5V cells (Fig.1b). We find that the Ca2+ influx elicited by acoustic waves was suppressed by >75% knockdown of Trpc1, Trpv4 and Trpm7, more specifically, 2- to 3-fold increase in Ca2+ response delay and >50% attenuation in excitation amplitude (Fig.1f). While Trpp2 knockdown revealed dissociation between the Ca2+ entry and acoustic wave stimulation. These data pinpoint Trpc1, Trpv4 and Trpm7 as the signaling sensor of acoustic waves by modulating Ca2+ influx in H5V cells.

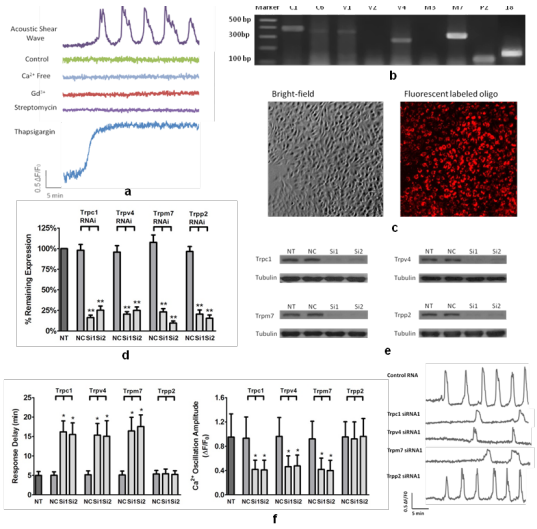

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Figure 1 Ca^{2+} influx pathways of Trpc1, Trpv4 and Trpm7 are activated by acoustic shear waves in H5V cells. a, Stretch-activated channels underlie Ca^{2+} excitation by acoustic waves. **b,** Expression of mechanical sensitive TRP channels in H5V cells by qRT-PCR analysis. C1, Trpc1; C6, Trpc6; V1, Trpv1; V2, Trpv2; V4, Trpv4; M3, Trpm3; M7, Trpm7; P2, Trpp2; 18S, 18S rRNA. **c,** Visualization of transfection efficiency in H5V. Transfection efficiency was monitored by the uptake of a fluorescence-based indicator, BLOCK-iT Alexa Fluor Red Fluorescent Oligo 72 hours post-transfection. It is shown that a transfection efficiency of over 90% could be achieved in H5V cells. **d,** mRNA knockdown percentage of Trpc1, Trpv4, Trpm7 and Trpp2 in H5V analyzed by qRT-PCR. NT, no treatment; NC, negative control RNA; Si1 and Si2, different siRNA sequences; error bars represent s.e.m.; double asterisk, P<0.01 versus the respective NC. **e,** Endogenous cellular proteins knockdown of Trpc1, Trpv4, Trpm7 and Trpp2 in H5V assayed by western blotting. **f,** Knockdown of Trpc1, Trpv4 and Trpm7 but not Trpp2 decelerate Ca^{2+} response and reduce Ca^{2+} oscillation amplitude in H5V cells. Asterisk, P<0.05 versus the respective NC.

Ca2+ Response to Acupuncture-induced Acoustic Waves in Cells: More Insights into the Roles of Transient Receptor Potential Channels (II)

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Introduction: Mechanosensation is ubiquitous and takes on many different forms, as cells, tissues, and entire organisms constantly receive and generate various mechanical stimuli. Previous works provide the direct link of acupuncture needle manipulation to intracellular Ca2+ signaling. Study I aims to unravel the underlying molecular mechanisms of this mechanotransduction in endothelial cells. In order to look for universal mechanosensitive channels to acupuncture-generated acoustic waves in various systems, we will further explore on another cell type: mouse embryonic fibroblast cells (NIH 3T3).

Methods: Materials and methods were as described in Study I. Sequence information for the Stealth RNAi duplexes is shown in Table 1.

Results:

Table 1 Stealth RNAi™ siRNA

Target Gene	Set of 2 Oligos	Primers (from 5' to 3')
Trpc1	siRNA1	Sense: UUAACAUUAUCAGAGCUGGACUGGC
		Antisense: GCCAGUCCAGCUCUGAUAAUGUUAA
	siRNA2	Sense: AUGAUUUGCUAUCAGCUGGAAGCUC
		Antisense: GAGCUUCCAGCUGAUAGCAAAUCAU
Trpv4	siRNA1	Sense: AGAAGCAGCAGGUCGUACAUCUUGG
		Antisense: CCAAGAUGUACGACCUGCUGCUUCU
	siRNA2	Sense: UAAUGGGCUCUACAGCCAGCAUCUC
		Antisense: GAGAUGCUGGCUGUAGAGCCCAUUA
Trpm7	siRNA1	Sense: AAUACUCUGUGACAACCUCAUCAGG
		Antisense: CCUGAUGAGGUUGUCACAGAGUAUU
	siRNA2	Sense: ACAAGUUGCUGACAAAUCUGACAUC
		Antisense: GAUGUCAGAUUUGUCAGCAACUUGU
Trpp2	siRNA1	Sense: AAACGAUGCUGCCAAUGGAGUGCUC
		Antisense: GAGCACUCCAUUGGCAGCAUCGUUU
	siRNA2	Sense: UUUACAGGCUGAAACUGCCAAGAGG
		Antisense: CCUCUUGGCAGUUUCAGCCUGUAAA

Similar mechanosensitive channel activities are also evident in NIH 3T3. Treatment with siRNA targeted against selected TRP channels show that Trpc1, Trpv4, Trpm7 but not Trpp2 trigger intracellular Ca2+ activation in response to acoustic shear waves. It is reported Trpc1 forms the stretch-activated cation channel in vertebrate cells[1], and Trpm7 is a stretch- and swelling-activated cation channel in human epithelial cells[2]. Trpv4 have osmotic sensitivity[3]. Trpp2 utilizes Trpp1 and Trpv4 to form a mechanosensitive molecular sensor, therefore Trpp2 alone may lack mechanosensitivity[3].

These two studies conclude that Trpc1, Trpv4, Trpm7 represent the stretch-activated cation channels serving as the acoustic wave sensor to initiate Ca2+ activation in endothelial cells and fibroblasts. As cytosolic Ca2+ is an important second messenger regulating diverse processes, this finding may help to explain the mechanism of acupuncture needle manipulation.

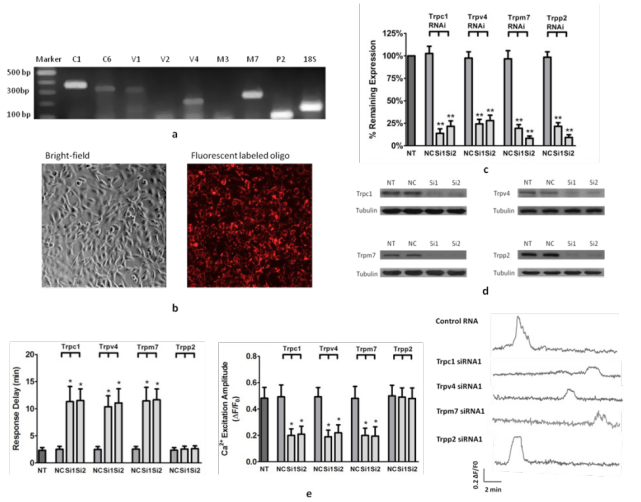

Figure 1 Ca^{2+} influx pathways of Trpc1, Trpv4 and Trpm7 are activated by acoustic shear waves in NIH 3T3 cells. a, Expression of mechanical sensitive TRP channels in NIH 3T3 cells by qRT-PCR analysis. C1, Trpc1; C6, Trpc6; V1, Trpv1; V2, Trpv2; V4, Trpv4; M3, Trpm3; M7, Trpm7; P2, Trpp2; 18S, 18S rRNA. b, Visualization of transfection efficiency in NIH 3T3 cells. Transfection efficiency was monitored by the uptake of a fluorescence-based indicator, BLOCK-iT Alexa Fluor Red Fluorescent Oligo 36 hours post-transfection. It is shown that a transfection efficiency of over 90% could be achieved in NIH 3T3 cells. c, mRNA knockdown percentage of Trpc1, Trpv4, Trpm7 and Trpp2 in NIH 3T3 analyzed by qRT-PCR. NT, no treatment; NC, negative control RNA; Si1 and Si2, different siRNA sequences; error bars represent s.e.m.; double asterisk, $P<0.01$ versus the respective NC. d, Endogenous cellular proteins knockdown of Trpc1, Trpv4, Trpm7 and Trpp2 in NIH 3T3 assayed by western blotting. e, Knockdown of Trpc1, Trpv4 and Trpm7 but not Trpp2 decelerate Ca^{2+} response and reduce Ca^{2+} excitation amplitude in NIH 3T3 cells. Asterisk, $P<0.05$ versus the respective NC.

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Quantitative imaging-based functional genomics screening in cancer research

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The metastatic spread of tumor cells is a dominant step in cancer progression which drastically reduces the disease prognosis. Our aim is to identify novel candidate metastasis genes that can be used as novel drug targets in cancer therapy. Invasion of cancer cells in the surrounding tissue is a crucial step in cancer metastasis and requires increased cell motility. Cell migration is a well orchestrated process that involves the continuous formation and disassembly of matrix adhesions, the structural anchor points between cells and the extra-cellular matrix. Still little is known about the molecular mechanisms that regulate adhesion dynamics during tumor cell migration, invasion and metastasis formation. We apply high throughput high resolution imaging-based functional genomics strategies to identify candidate genes that play a role in matrix-adhesion dynamics, tumor cell migration and 3D branching morphogenesis. So far we screened different siRNA libraries (kinases, phosphatases and adhesome) and a drugable cDNA library. This dynamic high throughput cell imaging together with multiparametric image analysis applied to relevant models enables us to understand the underlying signaling pathways that control tumor cell migration and cancer metastasis formation.

Kinome-targeted siRNA screen identifies kinases regulating the diffrentiation/ proliferation balance of prostate cancer cells

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We developed cell microarrays on extracellular matrix domain to perform massively parallel extinction of genes with siRNA and systematic screen kinase inhibition on cell lines and primary cultures of prostate cancer cells. We quantified the effect of the loss of each kinase on cell differentiation and proliferation, using novel high-throughput imaging and statistical analysis.

This functional screen, which produced a short list of new promising targets and siRNA currently under investigation, demonstrates that cell lines used as models for prostate tumors often displayed a different phenotypic outcome than tumor cells obtain from patients. Furthermore cell lines were less prone than healthy cells to siRNA-dependent systematic perturbation. Indeed functional redundancy in prostate cancer cells lines seemed to increase their robustness. Our results suggest than prostate cancer can be viewed as a robust system and open new perspective on therapeutic approaches.

DNA repair and transcription in living tissue

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Endogenous and environmental agents continuously damage DNA and directly interfere with proper functioning of the genome. Affected transcription resulted into imbalanced cellular homeostasis and is associated with accelerated ageing. Lesion-induced replication errors form the basis of genomic instability and are an important contributor to malignant transformations. Different DNA damage response (DDR) mechanisms, including diverse DNA repair and cell cycle control pathways protect organisms against the adverse effects of genomic insults. Inherited defects in DDR genes gives rise to a complex set of clinical features, including severe progeroid syndromes, extreme cancer-prone diseases or a combination of these.

Nucleotide Excision Repair (NER) is a versatile DNA repair mechanism that eliminates a variety of helix-destabilizing injuries, including UV-induced DNA damage. NER is a multi-step process requiring ~ 30 polypeptides and is intimately linked to transcription, illustrated by the pivotal role of the multi-subunit transcription factor, TFIIH, in both processes. Mutations in TFIIH are associated with a perplexing clinical heterogeneity, ranging from highly cancer-prone symptoms of xeroderma pigmentosum to premature-aging features of Cockayne syndrome, with a profound variable effect on organ functioning. Despite detailed knowledge of each of the separate mechanisms transcription and NER, little is known about the dynamic interplay and regulation between these processes and how different mutations in the same protein complex can cause such different clinical outcomes.

Spatio-temporal distribution, protein dynamics, protein-protein interactions and reaction kinetics of NER and transcription factors were determined in living cells by time-lapse imaging and different variants of FRAP (fluorescence recovery after photobleaching) on cells expressing key factors tagged with GFP. These studies revealed a stochastic and highly dynamic 'on the spot assembly' model of both processes.

However, these studies were performed within cultured cells, which are under constant stress and in a high proliferative state. It is unknown to which extent this highly dynamic organization holds for somatic tissues with differentiated and mostly post-mitotic cells. To allow in vivo analysis of transcription and repair in living tissues, we have created a knock-in mouse-model expressing fluorescently tagged TFIIH. YFP was targeted to the XPB (subunit of TFIIH) locus, generating a YFP-tagged TFIIH which expression is controlled by the endogenous regulatory elements. This mouse model allows studying NER and transcription kinetics in post-mitotic and highly differentiated cells embedded within their natural environment (tissues and organs). Moreover, genetic crosses with existing TFIIH mutant mouse-models allows the analysis of the fate of mutated TFIIH and its effect on repair and transcription in relevant (affected) tissue. A surprising difference in both the steady-state level and the distribution over distinct kinetic pools of TFIIH between different cell types was observed in living tissue.

An siRNA-based high-throughput microscopy screen to identify regulators of cell-matrix adhesion dynamics in breast cancer cells

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Introduction: Cell-matrix adhesions (CMAs) are dynamic multi-protein complexes that connect extracellular matrix to the actin cytoskeleton and play a crucial role in cell migration. Perturbation of CMA dynamics is critical in the process of cancer cell invasion and metastasis. Here we set out to identify genes involved in regulation of CMAs dynamics.

Methods: We performed a cell-based siRNA high-throughput microscopy screen and measured the effects of the siRNA-mediated knockdown on CMA dynamics. We used this approach for systematic screening of kinases (~700 genes), phosphatases (~200 genes) and CMA-associated proteins (~600; custom-made adhesome library) in human breast cancer MCF7 cells. Next to the loss-of-function in steady-state situation, we have also addressed the effects of gene knock-down on the assembly and disassembly of CMAs as two separate processes. The separation of these two processes was accomplished by applying nocodazole treatment followed by nocodazole washout, respectively.1 Subsequent automated quantitative analysis of the acquired confocal microscope images provided detailed information on the effects of candidate genes on different features of CMAs, including their size, shape, intensity and distribution within the cell. Correlating different CMA parameters will enable us to gain more insight in behavior and intrinsic properties of CMA (data mining) as well as to determine parameters, or the combinations of these, relevant for cell migration as such.

Results: We have completed the primary screen for kinases/phosphatases/adhesome libraries. In a secondary screen we have identified a set of ~50 siRNAs targeting either kinases or phosphatases that affect the assembly and/or disassembly of CMAs. We completed a secondary screen for these candidates using four individual siRNA duplexes. Approximately 70% of the hits are validated hits.

Conclusion: Hits from the secondary screen will be further evaluated in the context of (cancer) cell migration using various in vitro 2D and 3D assays. Hits whose knockdown/overexpression inhibits cancer cell migration in vitro will further be tested in in vivo breast cancer models. With these approaches we aim to identify new targets for anti-cancer therapy.

Acknowledgement: This reserach is funded by the European Community's Seventh Framework Programme.

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- IMAGING THE SPATIAL ORGANIZATION OF SIGNALLING NETWORKS
  Philippe Bastiaens – Dortmund, Germany
- SUBCELLULAR AND SUBPLASTIDIAL PROTEOMICS TO STUDY INTRACELLULAR TRAFFICKING OF PROTEINS
  Norbert Rolland – Grenoble, France
- 4D APPROACHES TO ANALYSE PROTEIN NETWORKS OVER SPACE AND TIME
  Marius Ueffing – Tübingen, Germany
- INTRACELLULAR SIGNALLING DYNAMICS OF HYPOXIA-INDUCIBLE FACTORS
  James Bagnall – Liverpool, UK
- INVESTIGATION OF THE DYNAMIC BEHAVIOUR OF P100 AND P105 IN SINGLE CELLS
  Denise Bakstad – Liverpool, UK
- DYNAMIC EVOLUTION OF ANGIOGENESIS DEPENDENT AND INDEPENDENT BRAIN TUMOR MODELS PRESENTING THE TUMORAL HETEROGENEITY OBSERVED IN HUMAN
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- META-ANALYSIS OF STRATEGIES FOR MATHEMATICAL MODELLING AND DATA INTEGRATION IN CANCER SIGNALLING SYSTEMS BIOLOGY
  Julio Vera – Rostock, Germany
- MODELLING MAPK CROSSTALK
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day four thursday february 11, 2010

Imaging the spatial organization of signalling networks

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Subcellular and subplastidial proteomics to study intracellular trafficking of proteins

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Introduction: Endosymbiotic evolution has resulted in the transfer to the nuclear genome of thousands of genes encoding the vast majority of chloroplast proteins. Most of these nuclear-encoded proteins are synthesized in the cytosol in the form of precursors. One important mechanism that participates in the regulation of the organelle biogenesis is the protein targeting that allows import of these precursors into the chloroplast. Two multiprotein complexes mediate this import process: TOC and TIC (Translocon at the Outer/ Inner envelope membrane of Chloroplasts). Until recently, all proteins destined for internal chloroplast compartments were thought to possess a cleavable N-terminal extension or transit peptide, and to engage this TOC/TIC machinery. Recent studies using proteomics and other approaches have revealed that this is not always true (1). Some reports have described alternative protein targeting routes to the envelope membranes. Other chloroplast (glyco)proteins were demonstrated to be transported through the secretory pathway (i.e. ER and Golgi apparatus) before their import into the chloroplast. These substrate-specific import pathways might play a role in the need to adapt to changes in developmental or environmental conditions, or to prevent competition effects between abundant and non-abundant precursors of chloroplast protein. However, and to date, no data are available at the scale of the complete chloroplast proteome.

Results: In combining their efforts to analyze the chloroplast proteome and reveal some alternative targeting pathways, members of the LPCV and LEDyP laboratories were significant actors of some of these recent discoveries (1). We recently went a step further into the knowledge of chloroplast proteins with regards to their accurate localization within the chloroplast. To achieve this goal, we first obtained highly pure chloroplast subfractions (i.e. envelope, stroma and thylakoids) and evaluated their cross-contaminations. Then, using a semi-quantitative proteomic approach, we assessed the partitioning of each protein in the three chloroplast subcompartments (2, 3, 4). This chloroplast accurate mass and time tags (AMT) database was also generated in such a way that it can be used to investigate the dynamics of the chloroplast proteome at the scale of a whole organelle.

Conclusion: The AMT strategy will be the basis of further quantitative studies (e.g. comparisons of chloroplast proteome from wild-type and mutant plants) aiming to decipher novel mechanisms regulating the protein targeting to the chloroplast.

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4D approaches to analyse protein networks over space and time

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Understanding complex cellular signal transduction networks is one of the big challenges in modern Biology. In spite of the abundance of protein interaction data, information on physiologically meaningful interactomes is limited. Proteomics is one of several possible ways to study protein properties on a large scale. Multimodal analytical approaches that catch the dynamics and physiological context are, however, needed to gain a more complex picture on the movie of life on the molecular level. Molecular imaging data can greatly contribute to the picture and systems biology approaches facilitate integration of heterogeneous data obtained from proteomics, molecular imaging and functional analysis.

Two examples of multimodal proteome-based approaches will be presented:

1) Catching the temporal and spacial dynamics of protein function can be of direct relevance to understand proteins and protein function in the context of human health and disease. We have taken an RNAi based proteomic and molecular imaging approach to generate hypotheses and assess the function of a yet enigmatic protein genetically associated with Parkinson's disease: Mutations in Leucine-rich repeat kinase 2 (LRRK2) are the single most common cause of inherited Parkinson´s disease (PD). Little is known about its involvement in the pathogenesis of PD mainly due to the lack of knowledge about the physiological function of LRRK2. To determine the function of LRRK2, we have identified LRRK2 protein complex components via quantitative immunoprecipitation combined with RNAi knockdown (QUICK). The resulting protein network indicates that the hypothetical biological function of LRRK2 is linked to neuronal vesicle transport along the cytoskeleton. To test this hypothesis we knocked down LRRK2 expression in cortical neurons. shRNA-mediated silencing of LRRK2 altered single vesicle kinetics and induces a redistribution of vesicles at the presynaptic site. As neurotransmitter containing vesicles are instrumental to neurotransmitter storage and release, LRRK2 mutations may affect the balance of vesicle dynamics needed for proper physiological function of these neurons.

2) We have systematically analyzed protein composition and interactions in photoreceptors, the light sensitive cells of mammalian eyes. Rhodopsin functions as light receptor in rods and was the first structurally resolved mammalian GPCR. By combining biochemical separation, affinity based isolation, mass spectrometry in combination with in silico modeling approaches we were able to define a protein network of more than 400 proteins associated with the structural and physiological organization of light reception and the actual signal transduction of light. Proteomic as well as structural information on the proteins participating in this hypothetical network was used and integrated allowing us to model ternary complex formation as well as the composition of macromolecular assemblies and its dynamic dissection into mutually exclusive complexes. Finally information regarding protein localization was added. The resulting network does not only offer an unprecedented view of signal transduction induced by this GPCR but also suggests important new functions, as well as displaying temporo-spacially regulated biological phenomena such as vesicle transport, cytoskeletal dynamics and dynamics in protein interactions regulating light adaptation.

Truly understanding cellular protein networks may still remain a goal far out. Yet, when combining the discovery of network components with methods that gather information on the topological distribution, dynamics and function of critical elements of a network, protein function can be assessed within its discrete systemic context of a living cell.

Intracellular signalling dynamics of Hypoxia-Inducible Factors

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Introduction: Regulatory signalling networks are often represented by static diagrams. However, observation of protein localisation and levels in single living cells have uncovered complex dynamics including oscillatory behaviour. We have previously shown the critical role of nucleo-cytoplasmic localization oscillations of the NF-κB transcription factor to control downstream pattern of gene transcription1. Here we have concentrated on the signalling system activated when cells are exposed to low oxygen (hypoxia). Hypoxia inducible factor (HIF) has emerged as a key regulator of the molecular hypoxic response, mediating a wide range of physiological and cellular mechanisms necessary to adapt to reduced oxygen. HIF orchestrates the transcription of hundreds of genes encoding proteins involved in energy metabolism, cell survival and cell death2.

Methods: We have used time-lapse confocal microscopy in hypoxic and normoxic conditions to image, with a high temporal resolution over a long period of time, fluorescent tagged HIF-1α and HIF-2α localisation in single living cells. We have also applied luminescence imaging to measure in real time and at a single cell level HIF-dependent transcription. A mathematical model describing the single cell responses to hypoxia is currently developed based on ordinary and delayed differential equations.

Results: We observed for the first time discrete single or repetitive pulses of HIF-1α and -2α accumulation in hypoxia (1% O2). The timing of the first peak response displayed a large heterogeneity and we have identified the cell cycle progression as being responsible for some of this variability for both HIF-1α and HIF-2α. The duration of HIF nuclear accumulation is robust and lasts ~240min. We performed real-time luminescence experiments using the HRE-luciferase reporter vector and we showed that the heterogeneity and transiency observed for HIFα accumulation was also present at the transcriptional level. We further developed a new mathematical model and showed that the PHD2 negative feedback loop was not sufficient to explain the complex dynamics observed experimentally. Indeed, the model predicts the presence of a positive feedback motif and/or involvement of oxygen independent regulations such as cell cycle progression that will need to be characterized in the future.

Conclusions: In conclusion, we observed a large number of distinct cellular behaviour, providing new levels of understanding of how cells detect low oxygen environment and translate it into output such as HIF nuclear accumulation and downstream gene transcription. Our quantitative data have allowed building a mathematical model for the HIF signaling system.

Acknowledgement: BBSRC funding for JB studentship and VS fellowship.

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Investigation of the Dynamic Behaviour of p100 and p105 in Single Cells

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Introduction: Nuclear Factor kappa B (NF-κB) is a transcription factor that regulates cellular stress and the immune response to infection. The classical heterodimer is composed of p65/p50 subunits and exhibits very well characterised nuclear-cytoplasmic oscillations (1). The less well-characterised p105 is the precursor protein for p50 and acts in a similar way to another NF-κB protein, p100, the precursor protein for p52. Both precursor proteins contain an NF-κB domain and an inhibitory IκB domain, which retains the proteins in the cytoplasm (2). The processing and movement of these two proteins is not as well characterised as p65. Single cell analysis in response to TNFα and IL-1β has been investigated by multi-parameter fluorescent time-lapse imaging.

Methods: SK-N-AS (human S-type neuroblastoma) cells were transiently transfected with pG-EGFP-p100-dsRedXP or pG-dsRedXP-p105-EGFP. Cells were imaged over 4 hours using a Carl Zeiss 510 confocal microscope in a humidified CO2 incubator (at 37 °C, 5% CO2) with a 63X 1.3 NA oil immersion objective. Cells were stimulated with 10ng/ml TNFα or 10ng/ml IL-1β with or without 10µg/ml cycloheximide.

Results: After treatment with either TNFα or IL-1β, it appeared that the dual labelled p105 was altered as a red fluorescent signal (indicative of p50) translocated to the nucleus of individual cells. This phenomenon occurred at a much faster rate with IL-1β.

When cells were stimulated with TNFα, dual labelled p100 gave similar results but at a much later time. However, after IL-1β treatment green fluorescence (indicative of p52) translocated to the nucleus and the red fluorescent signal (IkBδ) degraded at a much faster rate than in response to TNFα.

After cycloheximide and TNFα treatment, nuclear translocation of the green fluorescence (p52) was not observed indicating that de novo protein synthesis was required for this process. However, after cycloheximide and IL-1β treatment green fluorescence (p52) was observed in the nucleus suggesting that de novo protein synthesis was not required.

Conclusions: In single SK-N-AS cells, it appeared that IL-1β was able to activate the non-canonical pathway of NF-κB, as the green fluorescent part of a dual labelled p100 fusion protein translocated to the nucleus. Other imaging techniques, e.g. FRAP, FRET and FCS including cross correlation analysis, will be used to gain more information about the processing of these proteins in response to different stimuli. It will also be possible to investigate the activation of particular non-canonical genes after stimulation to further understand the NF-κB pathway in response to inflammation.

Acknowledgement: BBSRC, MRC

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Dynamic evolution of angiogenesis dependent and independent brain tumor models presenting the tumoral heterogeneity observed in human

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Introduction: Despite aggressive multimodal treatment strategies median survival of patients with glioblastoma is limited to 1-2 years. For the development of more efficient treatments, it is crucial to better understand the molecular processes of the disease progression such as deregulation of the cell cycle, neovascularisation, tumour cell migration and invasion. Molecular imaging will help to determine the dynamics of some of these important molecular alterations.

Our aim was to characterise using multi-modal molecular PET and MR imaging two new in vivo models of brain tumour development displaying two different phenotypes that could be observed in human patients.

Methods: Tumor tissues from two human glioblastomas were shortly maintained in culture as spheroids. Spheroids were stereotactically transplanted in the right brain hemisphere of nude rats (n=6 per group), and tumor development was followed from week 3 to week 8 after implantations. [18F]FDG-, [11C]MET-, and [18F] FLT-PET scans were acquired, together with magnetic resonance imaging (T2-weighted, and T1-weighted sequences before and after contrast agent injection; Average Diffusion Coefficient maps). Non-invasive observations were correlated with immuno-histochemical analysis.

Results: Two different tumor phenotypes have been obtained. The first group of rats, injected with spheroids derived from glioblastoma A, presented circumscribed tumors with extensive angiogenesis, peri-tumoral edema and necrotic areas. PET showed low uptake of [18F]FDG in the right brain hemisphere. Strong increase of [11C] MET accumulation was observed between week 3 and 4, while [18F]FLT uptake changed dramatically between week 4 and 5. The tumor was discernible already on the pre-contrast agent MR scans. The contrast was strongly enhanced after Gadolinium injection. Immuno-histochemical analyses showed high vascular proliferation. The second group of rats, injected with spheroids derived from glioblastoma B, displayed a highly invasive tumor, with neither signs of angiogenesis nor disturbed vasculature. Tumor development was revealed by a decrease of [18F]FDG uptake in the right brain hemisphere. Only very small increase of [18F]FLT and [11C]MET uptake could be observed at very late stage of the tumor development. The extent of the infiltrative tumors can be observed in T2-weighted MR images, but contrast is very low and no contrast enhancement could be observed in T1-weighted MRI after Gadolinium injection. Absence of angiogenesis can be explained by pre-existing blood vessels co-option as observed by immunohistochemistry on the brain section.

Conclusions: Multi-modal imaging is the basis for the determination of the dynamic behavior of tumor characteristics in vivo, and in particular for the description of its high heterogeneity. The angiogenesis-dependent glioma model resembles clinically well described imaging features and might serve as model for studying anti-angiogenic treatment. These clinically well described imaging parameters may miss diffusely infiltrative gliomas, or gliomas borders, which deserves the development of improved imaging parameters for diffusely infiltrating tumor cells.

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Meta-analysis of strategies for mathematical modelling and data integration in cancer signalling systems biology

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Rather than a strictly formalised methodological framework, systems biology is a flexible approach in which the modelling strategy used depends on a trade-off between the nature of the biochemical network investigated, the biomedical question to be elucidated and the quantity (and quality) of the experimental data available.

To further substantiate this idea, we selected a number of recent scientific publications in which systems biology was used in the context of cancer cell signalling. In our talk we will discuss and compare fundamental aspects of the strategy used to set up the mathematical models, integrate biomedical knowledge and quantitative data and analyse the system using theoretical and computational tools.

Modelling MAPK crosstalk

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Introduction: Cell movement is controlled by localised and coordinated activation of Rho family small GTPases. Whereas Rac and CDC42 are mainly activated at the front, RhoA activity is localised at the rear and front of the cell. Active RhoA signals are promoted downstream through the ROCK/LIMK cascade resulting in increased stress fibre formation and actin contractility needed for cell migration. More recently localised Rac and RhoA activities have been found to be highly cross-regulated in moving cells. The rapid changes between RhoA and Rac activities and their exclusivity suggest a switch-like mechanism.

Methods: Inhibition of MAPK signalling in Kras mutant cell lines results in a rapid increase in RhoA activity measured by biochemical methods and FLIM-FRET. Strikingly the negative regulation of RhoA by ERK signalling is observable on the single cell level, as pERK localisation and RhoA activity are mutually exclusive.

Proteomic and phospho-proteomic analysis revealed that a sub-set of RhoGEFs is directly phosphorylated by ERK. Results: ERK binds and directly phosphorylates GEF-H1 and P115RhoGEF. The phosphorylation reduces the exchange activity explaining the mutual exclusivity of RhoA and pERK. An un-phosphorylatable Serine to Alanine mutation not only increases global RhoA activity, but also severely impedes two and three-dimensional migration. Further the cell shape changes to a more rounded morphology. Additionally the correct polarisation of RhoA activity within the cell is lost. Conclusion: We propose that a negative feedback loop encompassing Rac, RhoA and ERK is responsible for the rapid switch between Rac and RhoA activity. Acknowledgements: AL, AVK, WK acknowledge Science Foundation Ireland and Cancer Research UK for funding, MRB acknowledges funding from a Marie Curie International Incoming Fellowship.

- IN TOTO IMAGING FOR THE RECONSTRUCTION OF EMBRYOGENESIS MULTISCALE DYNAMICS
  Nadine Peyriéras – Gif sur Yvette, France
- RECONSTRUCTING MULTI-SCALE DYNAMICS IN MORPHOGENESIS OF LIVING SYSTEMS
  Paul Bourgine – Paris, France
- SYSTEMS MODELING OF EPIDERMAL GROWTH FACTOR AND NERVE GROWTH FACTOR SIGNALING IN PC12 CELLS BASED ON INTERACTION PROTEOME DATA: NEW INSIGHTS INTO TRANSIENT VS. SUSTAINED ERK ACTIVATION
  Marc Birtwistle – Dublin, Ireland
- BIMODAL MOLECULAR IMAGING OF THE ENDOTHELIN-A-RECEPTOR EXPRESSION IN MURINE THYROID CANCER XENOGRAFTS USING SMALL ANIMAL PET AND OPTICAL IMAGING
  Katrin Büther – Münster, Germany
- FLUORESCENT MOLECULAR TOMOGRAPHY PROVIDES QUANTITATIVE SPATIO-TEMPORAL RESOLUTION OF SPECIFIC MOLECULAR PROCESSES AND DISEASES
  Karin Radrich – Neuherberg, Germany
- MULTIMODAL IMAGE DATA ANALYSIS AND QUANTIFICATION
  Maria Athelogou – Munich, Germany

day five friday february 12, 2010

In toto imaging for the reconstruction of embryogenesis multiscale dynamics

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Biological systems multiscale dynamics theoretical reconstruction should correlate genotype and phenotype, and integrate both upward and downward causation, thus providing the basis for simulating virtual organisms and powerful means for prediction and control. Achieving the phenomenological then theoretical reconstruction of biological systems multiscale dynamics first requires a paradigm of systematic investigation of the cellular behaviours and reconstruction of the cell lineage tree as a branching process in space and time. The 4D lineage tree is the framework to further integrate lower level dynamics (molecular and genetic) and higher level features (cell collective behaviours and system biomechanics). The main point in discussing these concepts is that so far biology largely escaped biological complexity. The mere accumulation of data from high throughput strategies does not readily lead to predictive models design and processes understanding. Gene network architecture and fate map annotation with gene expression data are not sufficient to achieve the reconstruction of processes dynamics. The latter requires gathering quantitative data from in vivo observation at the appropriate spatial and temporal scale for modelling gene network dynamics and integrating dynamical processes reconstructed at the molecular and cellular level of organisation. These strategies allow turning qualitative descriptions of biologists into more quantitative, formal descriptions amenable to automated treatment. At some point, we expect that Developmental Biology follows the path of high-energy physics: the strength and usefulness of phenomenological and theoretical reconstructions will come from the efforts of the scientific community to standardise and share their strategies, methods and open tools.

Reconstructing multi-scale dynamics in morphogenesis of living systems

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The next revolution in Biology and Biomedical applications will come from our ability to tackle biological systems complexity. This should be achieved within the framework of Complex Systems Science by reconstructing biological systems multiscale dynamics through the following scheme:

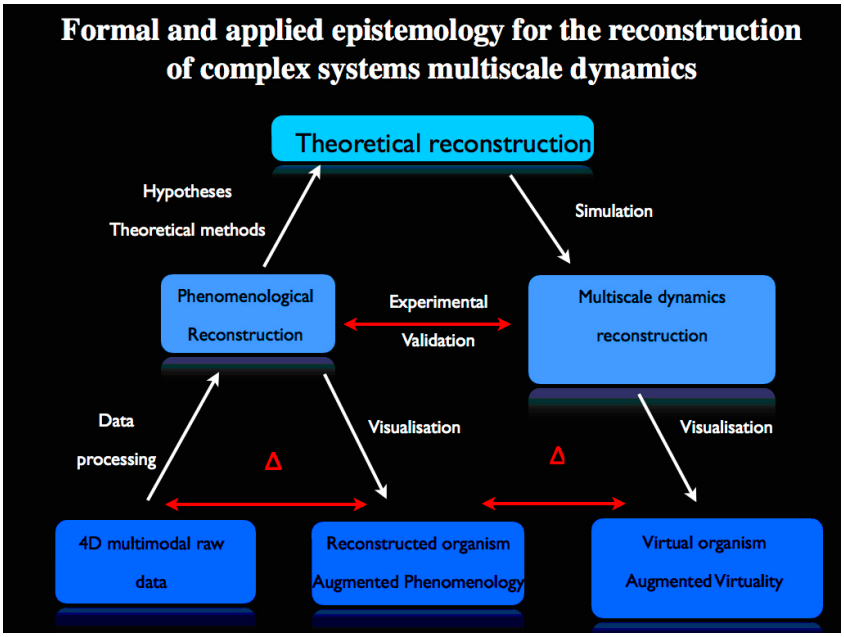

Fig 1: Formal and applied epistemology for the reconstruction of Complex Systems multiscale dynamics

This scheme means that explaining and predicting biological processes much further than actually achieved should come from the in vivo observation at all levels of organization. These observations should be performed with adequate spatial and temporal resolution and used to extract precise and accurate measurements providing what we call a phenomenological reconstruction. The latter is the basis for further theoretical reconstruction and simulation. Ultimately, dynamics at each level of organisation (molecular, genetic, cellular, multi cellular, systemic) should be integrated into a multiscale theoretical framework providing the reconstruction of the Physiome according to its international definition (Bassingthwaighte, International Physiome project).

The course will propose:
A general introduction about reconstructing symbolic dynamics. The Estimation Maximization method for phenomenological reconstruction of the tracking. The universalization of microdynamics for theoretical reconstruction of the macrodynamics.

Systems Modeling of Epidermal Growth Factor and Nerve Growth Factor Signaling in PC12 Cells Based on Interaction Proteome Data: New Insights into Transient vs. Sustained ERK Activation

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Introduction: Transient vs. sustained ERK activation in PC12 cells is a paradigm for how epidermal growth factor (EGF) and nerve growth factor (NGF) elicit distinct proliferation vs. differentiation cell fate decisions. Despite much effort to uncover how transient vs. sustained ERK dynamics arise, detailed mechanisms remain unclear.

Methods: To understand transient vs. sustained ERK signaling mechanistically, we have constructed an ordinary differential equation model of EGF and NGF signaling to ERK in PC12 cells that is trained, and thereby consistent with quantitative data from a variety of literature sources and our own experimental data. In particular, the basis of the model topology is largely derived from a quantitative proteomics screen (SILAC) for ERK interaction partners where we surveyed differential dynamic changes in ERK interacting proteins in PC12 cells treated with EGF or NGF.

Results: The emergent model structure suggests that only cell surface receptors can activate the PLCγ/PKC pathway, activation of which inhibits the key ERK cascade inhibitor RKIP and also activates Ras through the GRP family of GEFs. We furthermore uncover a novel positive feedback loop from ERK to NF1, the predominant Ras-GAP in PC12 cells. The main model prediction is therefore that rapid EGF-induced internalization of the EGF receptor and weak PLCγ activation, as opposed to surface retention of the TrkA receptor and strong PLCγ activation, generates the distinct ERK activation kinetics. Validation experiments show that increasing plasma membrane levels of active EGF receptor by stimulating with TGFα sustains the ERK response, whereas PLCγ knockdown makes the NGF induced ERK response transient.

Conclusions: We conclude that receptor compartmentalization and the PLCγ pathway play an important role in determining transient vs. sustained ERK signaling.

Acknowledgements: MRB acknowledges funding from a Marie Curie International Incoming Fellowship. WK, AVK, and MRB acknowledge Science Foundation Ireland and Cancer Research UK for funding.

Bimodal Molecular Imaging of the Endothelin-A-Receptor Expression in Murine Thyroid Cancer Xenografts using Small Animal PET and Optical Imaging

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Introduction: Endothelin (ET) receptor dysregulation has been described in a number of pathophysiological processes, including cancer. A number of human cancer cell lines exhibit an upregulated density of ET-A receptors, influencing tumor growth and aggressiveness. In thyroid cancer, elevated expression of the ET axis is reported for papillary and follicular tumor types. These tumors are mostly well differentiated, but some develop dedifferentiated fractions and these seriously influence prognosis of the patients. Radiolabelled ET receptor antagonists offer the possibility to noninvasively assess ET receptor distribution in vivo by positron emission tomography (PET). In addition, optical imaging is gaining increasing importance as a powerful technology for the preclinical study of diseases at the molecular level. Therefore, these diagnostic tools are invaluable for the evaluation of disease progression and therapy response. In this study, a radiofluorinated and a fluorescent biomarker, targeted to the ET-A receptor, were used for the evaluation of target expression in papillary thyroid carcinoma xenografts using small animal PET and optical imaging techniques.

Methods: Subcutaneous xenograft models of papillary thyroid carcinoma cell line K1 were established in CD-1 nude mice. Western blot and RT-PCR analysis were used to evaluate ET-A receptor expression of the tumor. A radiofluorinated analogue of the known ET-A receptor ligand PD 156707 was designed and applied in small animal PET experiments. Xenografts were evaluated using [18F]-fluoro-2-deoxyglucose ([18F]-FDG) prior to endothelin receptor imaging. In addition, a fluorescently labelled analogue of PD 156707 was also designed and used for optical imaging techniques such as fluorescence reflectance imaging (FRI) and fluorescence mediated tomography (FMT). Accumulation of the different tracers was evaluated using in-house software. Biodistribution studies were performed using FRI after optical imaging experiments.

Results: Using western blot analysis and RT-PCR the expression of the ET-A receptor on human papillary thyroid carcinoma was confirmed. Small animal PET experiments showed accumulation of the ET-A receptor targeted radiotracer in the tumor after 30-60 min. In optical imaging experiments using FRI and FMT techniques, a high fluorescence signal was visible in the tumor. After residual tracer washout via the kidneys and bladder (24-48 hrs) a tumor to muscle ratio of > 5:1 could be observed. Biodistribution studies after 24-48 hrs showed that 30% of total fluorescence was located in the lesion and about 10% in each kidney, the lung and the liver. Other organs showed a lower fluorescence signal.

Conclusion: Subcutaneous papillary thyroid carcinoma xenografts are a feasible model for the detection of endothelin receptor expression as a benchmark of tumor angiogenesis. Scintigraphic or optical imaging techniques like PET or FRI and FMT, respectively, in combination with radioisotope- or fluorescently labelled endothelin receptor ligands can be useful tools for the evaluation of target expression in tumoral lesions. Tracer kinetics, however, of radiotracers and fluorescent probes are very different. The optimal time point for PET measurements is in the range of 30-60 min, whereas optical imaging can ideally be performed after 24 hours.

Fluorescent Molecular Tomography provides quantitative spatio-temporal resolution of specific molecular processes and diseases

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Fluorescent molecular tomography (FMT) has proven to be a valuable technique for non-invasive monitoring of presence and role of molecules (proteins) in specific tissues of interest. For instance, its ability to resolve a variety of functions using either exogenously administered fluorescent probes or transgenic model organisms has enabled the identification of specific molecular processes and diseases. [1] [2]

This technology allows to three-dimensionally visualize deeper into the living animal by using multi-projection illumination and light measurements combined with mathematical models of photon propagation in tissue in order to reconstruct a quantitative image of fluorochrome biodistribution. [1] Current state of the art merges fluorescence and X-ray tomography to hybrid systems that are able to add anatomical information (X-ray) to functional investigations (FMT). [3]

We believe that FMT has the power of providing complementary data to systems biology models in comparison with more conventional experimental techniques. While traditional "-omics" approaches mainly consist of identifying the components making up a tissue or organism and - to a certain extent - their relationship, those approaches notoriously lack spatio-temporal and quantifiable information. [4]

Quantification is an important aspect in macroscopic fluorescent imaging through the determination of probe accumulation. Likewise, as experiments are carried out in-vivo, the natural environment of the investigated molecule is conserved. Thus, its spatial distribution, evolution over time as well as probe accumulation can be monitored simultaneously. Especially hybrid systems permit the determination of the exact position in space as well as a more reliable reconstruction through tissue segmentation. Further development towards multi-spectral imaging systems will allow for the investigation of several different molecules at once, improving therefore the comprehension of interrelationship, interaction, and mutual influence.

This talk emphasizes on these characteristics of FMT and shows that it is an up-and-coming technology for the systematic investigation of parameters relevant to accurate systems biology modeling.

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Multimodal Image Data Analysis and Quantification

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Introduction: The acquisition of imaging data in multiple modalities is becoming increasingly important in systems biology and medicine, in drug discovery and development, diagnosis, prognosis and treatment. Applying multiple image acquisition modalities to a single organ can provide invaluable structural and morphological information on different scales, dimensions and levels of resolution, providing a comprehensive understanding of cells, tissues, organs and organisms.

Method: Conducting cell- and tissue based assays in conjunction with tissue histology and in vivo medical imaging produces substantial volumes of digital data. The coordinated analysis of digital imaging data from multiple modalities can be challenging and labor intensive, but it is essential to the development of systems biology and medicine as for translational, personalized medicine and companion diagnostics.

We use a new image data analysis and quantification method, which is developed to overcome the challenges of multimodal image analysis. It is fundamentally different from conventional image analysis approaches and emulates human cognitive processes to extract information from digital imaging data. Rather than individual pixels, the technology identifies objects, examining them in context to draw inferences. This allows users to analyze images in 2D, 2D plus time, 3D, 3D plus time and multiple modalities using a single image analysis platform.

Results: To illustrate how the technology can be applied to a wide spectrum of multimodal image analysis tasks, image analysis applications for different modalities were developed for liver and breast. Image analysis results are presented for light-, fluorescence, confocal-, and electron-microscopy in 2 and 3 dimensions, cell-based assays and tissue probes, X-Ray, CT and MRI scans.

Conclusions: The presented examples for the multimodal analysis of liver and breast illustrate that this approach can be applied to other organs and organisms.

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- **poster one**
  A TRANSGENIC FLUORESCENT MAJOR SPERM PROTEIN REVEALS A ROLE OF MEMBRANE TENSION IN CYTOSKELETAL FLOWS IN NEMATODE SPERM CELLS
  Ellen Batchelder – Paris, France
- **poster two**
  CONTRAST AGENTS BOUND TO LDL-NANOPARTICLES. IN-VIVO ENHANCEMENT COMPARISON BETWEEN 1T AND 7T
  Giuseppe Del Grosso – Turin, Italy
- **poster three**
  VIMENTIN N-TERMINAL PHOSPHORYLATION SITE CLUSTER AS AN ENDOSOME REGULATOR
  Kimmo Isoniemi – Turku, Finland
- **poster four**
  BIORESPONSIVE MRI CONTRAST AGENTS BASED ON SELF-ASSEMBLING B-CYCLODEXTRIN NANOCAPSULES
  Jonathan Martinelli – Alessandria, Italy
- **poster five**
  ENZYME MEDIATED MRI PROBES: DESIGN, SYNTHESIS AND RELAXIVITY BEHAVIOUR OF A B-GALACTOSIDASE REPORTER
  Giorgio Pariani – Turin, Italy
- **poster six**
  MAGNETIC RESONANCE IMAGING & SPECTROSCOPY USING SPINNING COILS AND PORTABLE MAGNETS
  Dimitrios Sakellariou – Gif sur Yvette, France
- **poster seven**
  INVESTIGATING THE STIMULUS-DEPENDENCE OF THE NF-KB TRANSCRIPTION FACTOR SYSTEM USING FLUORESCENTLY – TAGGED PROTEINS
  Kate Sillitoe – Liverpool, UK

poster presentation thursday february 11, 2010

A transgenic fluorescent Major Sperm Protein reveals a role of membrane tension in cytoskeletal flows in nematode sperm cells

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Introduction: Sperm cells of nematodes like Caenorhabditis elegans translocate in a manner very much like other crawling cells, but their movement is dependent on the dynamics of Major Sperm Protein (MSP) rather than actin and myosin. Nematode sperm cells contain fewer proteins and cellular components, and thus present a simpler and unique comparative system with which to study cell motility. Methods: A new transposon insertion technique1 was used to generate worms expressing MSP N-terminally tagged with the photostable fluorescent protein tagRFP-T2 in sperm cells. Fluorescent dynamics in active sperm cells were observed by spinning disk confocal microscopy and photobleaching was performed. Kymograph and FRAP analysis were used to analyze the films.

Results: Patches of fluorescence are observed that move towards the cell body in both stationary and crawling active C. elegans sperm cell lamellipodia. Photobleached regions move rearward at the same speed as patches and do not recover fluorescence.

To investigate the role of polymerization in these flows, we altered membrane tension. Decreasing tension leads to a decrease in cell speed of adherent, crawling cells and reveals both membrane tension-dependent, and -independent components of cytoskeletal retrograde flows.

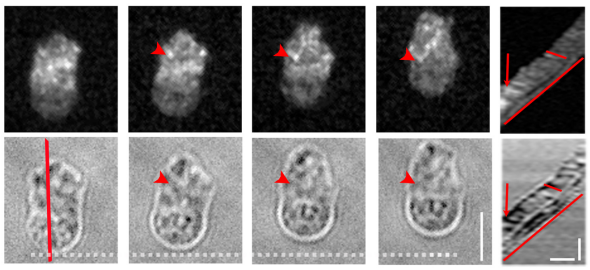

Fig 1. Still images from a time-lapse series of a crawling sperm cell containing tagRFP-T::MSP, with corresponding kymographs. Top: confocal fluorescence; bottom: transmitted light. Kymographs were created from the red line on the first image. Red arrow heads point to a feature undergoing retrograde flow, and the resulting streak made by this feature is indicated by an arrow in the kymograph. Example slopes for cell speed and retrograde flow are drawn in red lines on the kymographs.

Conclusions: Retrograde flow has been observed in actin-containing cells; we show that it is also a conserved feature in the different biochemical context of the MSP cytoskeleton. In actomyosin-driven cells, retrograde flow is thought to result from resistance of the plasma membrane against polymerizing filaments and myosin contraction at the rear of the cell. Since one component of flows in C. elegans sperm cells is independent of membrane tension/polymerization and no molecular motors have been described in this system, we propose disassembly of the MSP network as a novel driving force for retrograde flow.

Slowed cell speed upon a decrease in membrane tension is not predicted by the Brownian Ratchet model of cell motility, but is a hallmark of blebbing cell motility. However C. elegans sperm cells do not exhibit signs of blebbing. We propose instead that membrane tension affects intracellular pressures and flows that deliver MSP subunits to growing filaments, and therefore affects polymerization and crawling speed (an idea developed in3). Since disassembly is not affected by membrane tension, this component of retrograde flow remains unchanged.

Acknowledgements: We acknowledge Vincent Frasier of the PICT-IBiSA Imaging Facility at the Institut Curie for microscope use and support.

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Contrast Agents Bound to LDL-Nanoparticles. In-Vivo Enhancement Comparison between 1T and 7T

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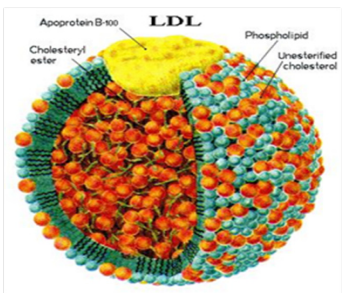
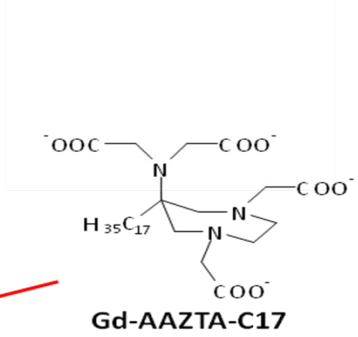
Introduction In respect to other molecular imaging modalities such as PET or SPECT, the low sensitivity is the main limitation of the Magnetic Resonance-Molecular Imaging (MRMI) approach. Therefore, the success of a MR-Molecular Imaging protocol strongly relies on the amplification effects associated to the accumulation of the agents at the pathological site. To this purpose the use of nanoparticles as carriers for MRI contrast agents (CA) has both the advantage to transport a high number of CA units at the site of interest and an improved efficiency of their contrast enhancement properties. In fact, macromolecular contrast agents show a relaxivity peak centered at 0.7-1T as a consequence of their slow tumbling time that causes a relaxivity increase of about three-four times with respect that measured at higher fields used in molecular imaging applications (3-7 T). Low Density Lipoproteins (LDLs) are naturally occurring nano-sized systems (22 nm diameter) transport cholesterol to cells expressing the LDL receptor. These receptors are overexpressed in tumor cells, LDL loaded with Gd complexes may be considered good probes for the MRI visualization of tumors. In the present work the highly sensitive Gd-AAZTAC17 complex was used to label LDLs.

Methods The cellular uptake of the Gd-labeled LDL was first tested in vitro on HepG2 and B16 tumor cells line. In vivo, Gd-labelled LDLs were administered to mice subcutaneously inoculated with B16 cells line. MRI was performed at 1 (Aspect M2) and 7T (Bruker Avance 300) before, 5 and 24 hr post-contrast injection.

Results Gd-AAZTAC17 binds strongly to LDLs yielding a relaxivity of 22mM-1s-1 (at 20 MHz) for the supramolecular adduct. About 300 molecules of the lipophilic agent can be loaded per LDL unit. The NMRD profile of the adduct is characterized by a relaxivity peak typical of slowly moving systems. MRI analysis, performed 5 hours after the injection of Gd-AAZTAC17 on C57 mice bearing transplanted melanoma tumor (B16), showed a significantly higher tumor signal intensity enhancement (~110%) at 1T with respect 7T (~15%).

Conclusions LDLs act as efficient carriers for the delivery of the lipophilic Gd-AAZTAC17 to tumor cells. To acquire MRI images at lower field (1T) results advantageous using macromolecular imaging probes such us Gd-labeled LDL in the detection of tumor cells both "*in vitro*" and "*in vivo*".





Gd-AAZTA-C17

Vimentin N-terminal phosphorylation site cluster as an endosome regulator

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Introduction: Vimentin is the major type III IF protein in mesenchymal cells. It has been shown to have roles in cell adhesion, migration, and signaling by acting as an integrator and organizer of regulatory and signaling proteins. Phosphorylation of vimentin on the N-terminus has been shown to have roles in regulating filament organization, mitosis, cell migration, and smooth muscle contraction. In particular, we have studied in vivo function of N-terminal serine cluster phosphorylation sites (Eriksson et al., 2004) previously shown to have roles in β1-integrin recycling by site-directed mutagenesis (phosphomimetic Asp and phosphorylation-deficient Ala).

Methods: Vimentin negative MCF7 human breast adenocarcinoma and vimentin positive HeLa cervical cancer cells were used. Plasmid constructs used were cloned in pCDNA vector or received from Johanna Ivaska's lab. Cells were fixed and labeled for confocal microscopy imaging 2 days after electroporation transfection. For flow cytometry analysis the cells were analyzed 2 days after electroporation. For flow cytometry cells were incubated with 75 µg/ml 70 kD FITC-dextran or with folate-covered ~200 nm nanoparticles for 6 hours.

Results: The phosphomutants of vimentin serine cluster (S4,6,7,8,9A and S4,6,7,8,9D) at the N-terminus form vesicle-like structures inside HeLa, MCF7 and SW13 cells. Confocal microscopy analysis reveal that structures formed by vimentin S4,6,7,8,9A appear small in the cell periphery, but grow and become greatly enlarged and noticeably hollow towards the cell center. Using endosome markers we have found that vimentin S4,6,7,8,9A mutant localizes over certain endosomal compartments. The early endosome marker EEA1 and late endosome marker Rab7 have been found in some vimentin-covered vesicles in MCF7 cells. On the contrary, early endosome markers HRS, 2xFYVE and Rab5 and lysosome marker LAMP-1 did not appear inside vimentin-covered vesicles. Additionally, EEA1 and Rab7 continuously appeared in the large vesicles. Endocytosis experiments indicated that folate-covered nanoparticles and FITC-dextran were significantly less endocytosed in Hela and MCF7 cells containing vimentin S4,6,7,8,9A than for wild-type vimentin. Quantification of structures formed by vimentin S6, S4,6, and S4,6,7,8,9A and D showed that vimentin S4,6A and even more S4,6,7,8,9A produced large amounts of endosomal formations but not vimentin S6.

Conclusions: Colocalization studies suggest that dephosphorylation of vimentin N-terminal serine cluster distrupt vimentin filamentous form and causes vimentin to cover specific early and late endosomes, but not lysosomes. Large vesicular structures suggest a possible defect in protein transport or vesicle dynamics.. Flow cytometry data suggests that phosphorylation/dephosphorylation of the vimentin N-terminal serine cluster has an effect on both receptor and non-receptor-mediated endocytosis. Quantification of typical aggregates in different phosphomutants of vimentin N-terminal serine cluster suggests that endosome-regulating phosphorylation-dephosphorylation of this cluster has a complex mechanism including S4 as an important player.

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Bioresponsive MRI contrast agents based on self-assembling β-cyclodextrin nanocapsules

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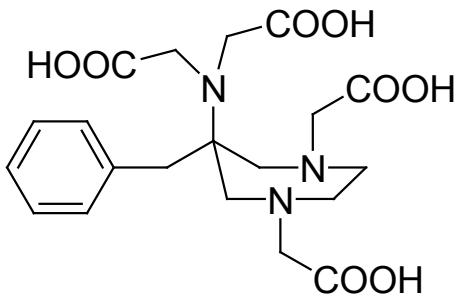
Introduction: One of the most exciting areas of research about MRI contrast agents is that concerning responsive or "smart" ones, whose performance is modulated by changes in physiological environment such as pH, partial oxygen pressure, metal ion concentration, enzyme activity etc.

We have designed and synthesized a new type of contrast agent containing disulfide bonds and thus sensitive to reducing conditions like those where specific enzymes or high radical concentrations are associated with a disease state (e.g. tumors, strokes etc.). They are meant to be MRI silent until activated by a reducing environment, which can "switch on" the high relaxivity.

Methods: The macromolecular architecture of the agent is based on nanocapsules prepared from perthiolated β-cyclodextrins via oxidation of the thiol groups to form S-S bridges. The assembly was carried out in the presence of a 5:1 excess of Gd-benzyl-AAZTA as the MRI-active paramagnetic complex, able to form inclusion adducts with the cyclodextrin building blocks and thus being "trapped" inside the capsules during their formation. After purification of the nanomaterials and removal of the external Gd-complexes through prolonged dialysis, the macromolecular systems were characterized via relaxometric analyses.

Results: The NMRD profile confirmed that a significant amount of complex is caged within the structure, but with a limited water exchange at the metal centers. Dynamic light scattering measurements also showed that the size of the nanocapsules prepared in the presence of the Gd-complex is sensibly bigger than in its absence (120 nm vs. 30 nm).

Conclusions: β-cyclodextrin nanocapsules containing Gd-complexes appear to be promising as MRI contrast agents responsive to reducing biological environments. Experiments of cleavage of the disulfide bonds by reducing agents such as mercaptoethanol or dithiothreitol are expected to lead to a remarkable increase of the water proton relaxation effect, since the disaggregation of the nanocapsules implies the release of the "trapped" Gd-complexes, that in this way become able to exchange water with the bulk.



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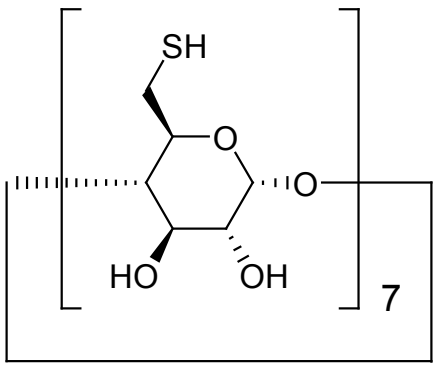

per-6-thio-b-cyclodextrin

Enzyme mediated MRI Probes: Design, Synthesis and Relaxivity behaviour of a β-galactosidase reporter

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Introduction: The efficiency in accumulating imaging reporters at the targeting sites is the major task in MRI for the visualization of biological processes at the cellular and molecular level.[1] One way to tackle this amplification route is to seek for the formation of self-assembled aggregates of Gd-chelates. Bogdanov et al. showed that melanin-like polymers can form when hydroxo-functionalized Gd-chelates are in the presence of the suitable enzymes (e.g. tyrosinase or myeloperoxidase).[2] Our goal is to exploit this approach in order to set up a MRI method to assess the expression of β-galactosidase (β-gal).

Methods: Relaxometric methods have been used for the in vitro physico-chemical characterization of the Gd-based probe either through the measure of NMRD profiles over a frequency range from 0.01 to 70 MHz and at a fixed frequency value of 20 MHz on Stelar Relaxometers (Mede – Italy). The efficiency of responsiveness to β-gal and tyrosinase enzymes has been assessed through relaxometric and spectrophotometric measures either in buffered water solutions and in cellular systems (B16F10 – murine melanoma cell line). MR Images of capillaries containing the cell pellets, were recorded on a Bruker Avance300 spectrometer operating at 7.1T equipped with a microimaging probe and on an Aspect spectrometer operating at 1T using a standard T1 weighted multislice multiecho sequence.

Results: A Gd-DOTA based probe containing a tyrosine –OH functionality protected by a galactose moiety (Gd-DOTAgal) has been synthesized and relaxometrically characterized. Upon cleavage of the galactose moiety (step activated by the presence of β-gal) the tyrosine group becomes available for the tyrosinase activated melanin-like polymerization. It is well established that the relaxivity of Gd-complexes increases if they are part of macromolecular systems as a consequence of the lengthening of their reorientational correlation time (τR). We exploited this concept to prove that the tyrosinase enzyme acts on the Gd-DOTAgal complex, previously activated by β-gal cleavage, fostering the formation of a melanin-like high relaxivity aggregate. The in vitro relaxivity of the target molecules with and without sugar has been investigated by 1H NMRD in the presence and the absence of enzymes. The enzymatic responsiveness has been assessed in vitro either in β-gal/tyrosinase containing water solutions and in B16F10 murine melanoma cell lysates as this cell line has been reported to possess high tyrosinase activity related to its natural melanin pigmentation. Further preliminary invesigations have been carried out on a related Gd-DOTA based probe cotaining a cis-diol functionality in order to obtain a system that spontaneously polymerizes once activated by β-gal cleavage without the action of tyrosinase enzyme. This second system enlarges the applicability of the →β-gal reporter probe to a wider collection of cellular systems other than melanoma ones.

Conclusions: The Gd-DOTAgal derivative has been proven to be a good β-galactosidase expression reporter that results in a system that can undergo polymerization in the presence of Tyrosinase upon cleavage of the tyr-gal bond.

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Magnetic Resonance Imaging & Spectroscopy using Spinning Coils and Portable Magnets

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Introduction: There are many cases where the sample, object or subject under investigation, have spatial requirements that cannot be fulfilled with standard superconducting MRI magnets and the need for portable devices becomes apparent. Additionally, MR technology can in principle be applied for microscopy where molecular imaging of metabolites in micro-biopsies could offer an important amount of information, but the spectral broadening due to anisotropies hides information [1].

Methods: We first present an approach to portable magnet design based on an analytical theory that allows the design of arbitrary magnetic fields inside and outside of a permanent magnet [2]. The control of the field profiles can be mastered at any order, which leads to large regions of interest and deep object-penetration distances.

Secondly, we introduce the rotating micro-coils, as a means to achieve sensitive detection, high-resolution spectroscopy [3] as well as ultra high-resolution 2D and 3D images of microscopic objects, using the stray field of the magnet [4], and sample spinning, which averages anisotropy related effects.

Results: e will present the first high-uniformity, linear-gradient (3.3 T/m), one-sided permanent magnet system, which was conceived using our theoretical framework. Measurements show that the magnitude of the field is 0.33 T and its profile is linear up to 5 ppm inside a 10 mm diameter spherical volume, positioned 20 mm from the surface of the magnet. The field can penetrate up to 5 cm inside an object with sufficient uniformity (3%) for diffusion measurements.

We will also present images obtained using magic-angle coil spinning of water phantoms, silicon carbide samples as well as mouse bone and tooth, in the presence of stray field gradients up to 5 T/m offering extreme spatial resolutions (9 mm by 35mm).

Conclusions: Our novel theory is a powerful tool for designing MRI magnets, which can be easily optimized for organ-specific studies or for multi-modal imaging applications. The introduction of solid-state methodology in MR microscopy shows that high-resolution images can be obtained without the need of pulsed field gradients. Both approaches could in principle be combined, by magnetic field rotation, which is currently underway in our Laboratory.

Acknowledgement: Our group has received funding from the European Research Council under the European Community's 7th Framework Programme (FP7/2007-2013), ERC GA # 205119, IIF GA # 237068 and from the Agence Nationale de la Recherche contrat ANR-06-JCJ0061. We are also grateful to Dr. Sara Laurencin Dalicieux (Toulouse, France) for providing the mouse tibias bone.

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Investigating the stimulus-dependence of the NF-κB transcription factor system using fluorescently – tagged proteins

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Introduction: The NF-κB signalling network is a complex system of transcription factors and their regulators many of which are dynamically controlled by their localisation or degradation. Single cell imaging of network components allowed an examination of the kinetics of these processes. There were differences in the regulation of the transcription factors depending on the co-expressed inhibitor and the stimulus.

Methods: Single cell confocal microscopy was used to image various fluorescently-tagged NF-κB system proteins in transiently transfected mouse embryonic fibroblasts. Time lapse imaging using a Zeiss LSM 510 confocal microscope in humidified conditions at 37°C 5% CO2 was used to observe changes in fluorescent protein levels and localisation. Cells were stimulated with either TNFα (10ng/ml) or LPS (0.5µg/ml).

Results: Different stimuli give rise to different dynamics of the NF-κB, p65 (p65-dsRedXP) transcription factor translocation from cytoplasm to the nucleus and back. The inhibitors IκBα, IκBβ and IκBε (which regulate the cytoplasmic localisation of the p65 protein) degraded with different dynamics depending on the stimulus applied. The co-expression of p65 and different IκBs allowed the observation that these endogenous inhibitors play a more complex role than previously thought. The inhibitors slowed down (or counter-intuitively) increased the p65 nuclear translocation rate in a stimulus-dependent manner. Other negative regulators of the system such as A20 also altered p65 translocation dynamics in response to some stimuli but not others.

Conclusions: The timing of p65 translocation and potential for continued nuclear oscillations in p65 localisation were both signal-dependent characteristics of the response.

IkBs can exert differential modulatory effects on the translocation response of the NF-kB protein p65 to speed up or slow down it's nuclear translocation.

The signal-specific responses of cells may be determined in part through the generation of different p65 translocation profiles.

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