Session I: Flow sorting

Methodological aspects of gene expression in high-speed flow sorted stem cells (side population cells) from mouse bone marrow

Oleg Tsinkalovsky. *Stem Cell Research Group, The Gade Institute, Department of Pathology, Haukeland University Hospital, Bergen, Norway* (oleg.tsinkalovsky@gades.uib.no)

Since little is known of gene expression in hematopoietic stem cells related to time, and in particular as compared to the total bone marrow population, this has been addressed in B6 D2f black mice. The aim was therefore to establish if clock genes are expressed in stem cells - side population (SP) cells and if they exhibit a rhythmic pattern in the same way as other cell types. We have therefore done a comparison between the expression of clock genes in the whole marrow and in mice kept under standardized conditions. Bone marrow was obtained from the femurs of every 4 hours for 24-hours periods. Following Hoechst 33342 fluorescent labelling, the SP containing early progenitor cells was highly enriched by the ultra-speed flow cytometric cell sorting using the MoFlo instrument. The transcription of 7 different genes Per 1, Per 2, Bmal1, Clock, Cry 1, Rev-erb alpha and Cyclin D3 was measured with real-time PCR and compared to several reference genes (18S, GAPDH and 36B4). Some preliminary data on clock genes expression are discussed.

Flow cytometric sexing of mammalian spermatozoa

Preben Christensen*. Department of Production Animals and Horses, Section for Reproduction, Royal Veterinary and Agricultural University, Dyrlaegevej 68, 1870 Frederiksberg C, Denmark. Tel: +45 35 28 29 70* (PC@kvl.dk)

Beeing able to preselect the sex of offspring at the time of conception ranks among the most sought-after reproductive technologies of all time. Sex preselection that is based on flow cytometric measurement of sperm DNA content to enable sorting of X- from Y-chromosome-bearing sperm has proven to be reliable with many species at greater than 90% purity. Offspring of the predetermined sex in both domestic animals and human beings have been born using this technology since its introduction in 1989.

The method involves treating sperm with the fluorescent dye, Hoechst 33342, which binds to the DNA and then sorting them into X- and Y-bearing sperm populations with a flow cytometer modified specially for sperm. With the advent of high-speed cell sorting technology and improved efficiency of sorting by a new sperm orienting nozzle, the efficiency of sexed sperm production is significantly enhanced. Under typical conditions the high-speed sperm sorter with the orienting nozzle (HiSON) results in purities of 90% X- and Y-bearing sperm at 18 million sperm per h for each population. This represents a 50-fold improvement over the 1989 sorting technology using rabbit sperm.

Today, sexed sperm are commercial available for cattle. Initial experiments for this species involved deep intra uterine insemination with low dose (2 million sperm) but more recent experiments with normal intra uterine inseminations have shown similar success rates. In comparison to conventional insemination with 15 million sperm, pregnancy rates are still approximately 10 percentage units lower. At present, it is unclear if this reduction in fertility is related to the difference in number of sperm per dose or reflects damage to sperm during sorting. Up to now, several thousands of healthy calves have been born after this technology.

The method have also been applied for other species such as sheep, horses, exotic and endangered species and have in some cases been used in combination with in vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI). The most important application of sexing in the human population is to minimize sex-linked genetic disease. A more controversial application is sex selection for purposes of family gender balancing.

Cell surface expression of PrP^C on subsets of peripheral blood cells from sheep of different genotypes

Jakob Ohm[1], Martha J. Ulvund[2], Ulla Riber[1], Jens S. Andersen[1], Peter Lind[1] Jørgen K. Larsen[3] and Peter M.H. Heegaard[1]

[1)]Danish Institute for Food and Veterinary Research, Department of Veterinary Diagnostics and Research, Bülowsvej 27, 1790 Copenhagen V, Denmark. [2)]Norwegian School of Veterinary Science, Kyrkjevegen 334, 4325, Sandnes, Norway. [3)]Finsen Laboratory, Strandboulevarden 49, 2100 Copenhagen Ø, Denmark (jakobohm@vetinst.dk)

It is well established that different genotypes of sheep differ widely in their susceptibility to development of scrapie, a sheep disease of the Transmissible Spongiform Encephalopathy (TSE) family caused by aggregation of misfolded proteins in the brain. The reasons for the differences in susceptibility are not known but are obviously important with regards to both the understanding of the pathogenesis of scrapie and for the development of means to control scrapie and other prion related diseases. The pathogenic prion protein (PrP^{Sc}) is furthermore thought to be in close contact with the normal prion protein (PrP^C) during the conformational change of PrP^C to PrP^{Sc}. With a firm establishment of scrapie infectivity present in blood from sheep terminally ill with scrapie, blood PrP^C became an interesting aspect. We therefore undertook a study to elucidate the connection, if any, between the level of PrP^C expression and expression pattern on the surface of blood cells and the susceptibility/resistance towards scrapie. Here we show a correlation between scrapie susceptibility and cell surface expression of PrP^C on sheep blood monocytes, gamma delta T-cells and B- cells.

Cell Sorting with Pathogens - Safety Precautions in Flow Sorting at Containment Level 3

Julie Rogers. *Health Protection Agency (Porton Down), Salisbury, Wiltshire SP4 OJG, UK. Tel: +44(0) 1980 612866* (julie.rogers@hpa.org.uk)

Flow cytometry staff at the HPA Porton Down site have several years of experience in providing flow cytometry services at containment level 3 (CL3). This, combined with the experience on site of biosafety and of specialist microbiological facilities, has allowed the development of a contained sorting capability. The requirement is for high speed sorting of a range of bacterial pathogens, including *Mycobacterium tuberculosis* and *Bacillus anthracis*.

After assessment of a number of flow cytometers, the MoFlo was considered the most suitable and technically adaptable sorter for housing in containment. A flexible film isolator was determined to be the only feasible option to allow CL3 work to be conducted under UK Heath and Safety requirements that demand primary containment of hazards. Siting of MoFlo components, the isolator design and installation of facility services have been implemented to maintain ease of working whilst minimise the risks to the operator.

Rigorous procedures are required for the safe operation, cleaning, and decontamination of both the equipment and the facility. These steps are required to protect both the operators and the service personnel. Two non-pathogenic organisms were selected to validate the containment systems. *Bacillus subtilis* var niger was used to simulate a bacterial agent and *Escherichia coli* (NCIMB 9481) MS2 coliphage was used to simulate a virus. The expected bioburden to be released during operation and following blockage of the nozzle was determined by aerobiological assessment.

Biosafety considerations are currently being highlighted across the field of flow cytometry. Our experience in working in a specialist containment level 3 facility provides insights into issues that are relevant to cell sorter operators working with all types of samples. These approaches to containment apply generally and would address safety issues such as those involved with sorting virally infected mammalian cells, tumour cells etc.

Session III: Immunomagnetic sorting

Immunomagnetic cell sorting - comparison of methods

Jan Pravsgaard Christensen. *Institute of Medical Microbiology and Immunology, The Panum Institute, University of Copenhagen, Copenhagen* (j.pravsgaard@immi.ku.dk)

Purification of cells is often used to demonstrate specific functions of a cell subset. This purification can be achieved either by flow sorting or by separation with magnetic beads. Several considerations must be taken into account before choosing the appropriate methods for separation. Beside the obvious point of availably instruments, some points to think about are price, no. of separations, no. of parameters needed for separation, quality of cells and purity. Although the purity generally are lower with magnetic beads compared to flow sorting, many assays work quite well, and may even give the same results, compared to sorting. Examples of how we have used magnetic beads in our studies will be demonstrated.

Session IV: Laser microdissection

Laser microdissection in proteomics

Pavel Gromov. *Institute of Cancer Biology, Danish Cancer Society, Copenhagen* (psg@cancer.dk)

Presently, the application of state-of-the-art technologies from proteomics and functional genomics to the study of cancer is rapidly shifting to the analysis of clinically relevant samples such as biopsy specimens. Studies to identify markers of disease or protein changes that are informative for disease progression must address the problem of tissue heterogeneity that represents one of the most important hurdles we face today for implementing the new technologies. Laser microdissection is now well established as a tool facilitating the enrichment of cells of interest from tissue sections overcoming the problem of tissue heterogeneity.

For any laser microdissection methods, the number of cells required to obtain a 2D protein profile suitable for protein detection and mass spectrometry identification is in the order of 50,000 cells or more, a fact that hindered the use of this technology on a routine basis. Even if a smaller number of cells would be required thanks to more sensitive protein detection procedures one would still need to address the problem of cellular heterogeneity, as immunohistochemistry with a single antibody marker can often detect heterogeneity even in ducts that are composed of a small number of cells. Here we discussed several aspects of laser microdissecton technique (P.A.L.M. Microlaser Technology) as applied to the gel-based proteomic analysis of breast cancer.

Laser mediated live cell handling: Detection, isolation and capture of single live cells by Laser Microdissection and Pressure Catapulting (LMPC)

Rainer Gangnus and Renate Burgemeister. *P.A.L.M. Microlaser Technologies AG, Am Neuland 9+13, 82347 Bernried, Germany* (www.palm-microlaser.com)

Modern molecular research relies on the capability of getting access to pure samples. Laser Microdissection and Pressure Catapulting (LMPC) is a well-known method to isolate and collect specific cells from complex tissues for subsequent molecular analyses. Tissue preparation and extraction protocols allow the utilization of microsamples for quantitative molecular analyses like, e.g., PCR and RT-PCR amplification, microarray analysis, and MALDI/SELDI spectrometry. Up to now, LMPC mostly has been applied on paraffin and cryosections, cell smears, cytospins and chromosome preparations.

An important innovation is the laser driven isolation of live cells out of a cell culture. Individual or small groups of cultured cells can be used for direct molecular analysis or re-cultivation. This helps scientists to isolate cell clones and separate different cell types by morphology or fluorescent label. The work with selected live cells is extremely facilitated with this new approach and opens a wide field of new applications and research possibilities in molecular biology and medicine as well as cell biology.

The Principle of LMPC (Laser Microdissection and Pressure Catapulting) is a pulsed UV-A laser coupled into a routine research microscope and focused via the objective lenses to a micron-sized spot diameter. Within the narrow laser focal spot forces are generated that allow ablation of material (that is cutting; Laser Microdissection), whilst the surrounding tissue remains fully intact.

Using the same laser the separated cell(s) or selected tissue area can be lifted up (Laser Pressure Catapulting) and captured in a collection device. This is a totally non-contact process, as only focused light is used for the transportation of a selected area into a collection device. Targets, from parts of chromosomes up to an entire living organism, as the nematode C. elegans, are successfully transported without impairing the biological information or the viability of the specimen.

The same principle is applicable for the collection of live cells from a cell culture.

The catapulted material subsequently will be spun down and analyzed, or used for further experiments.

There is high interest in new methods to handle single live cells. For example stem cell isolation, selective ablation of unwanted cells in a cell culture, creation and maintenance of mixed cell cultures, and maintaining specific cell type ratios in mixed cultures in general is very hard work in cell biology. With the development of a protocol to select and collect (even single) live cells in a non-contact way that kind of work will be dramatically simplified and accelerated.

Using the easy to handle protocol of catapulting live cells allows getting access to clearly selected single or few cells for, e.g., all kind of cloning experiments. This positive selection allows catapulting of desired cells and their re-culture. Negative selection, this means elimination of unwanted cells in a cell culture, is done by ablating undesired cells from a mixture and ongoing culture of only the remaining cells. This way it is easy to obtain homogeneous cell populations.

Besides that, the laser can cut the cell membrane of mammalian cells, or drill holes into the solid wall of plant cells. Even within live cells entire organelles, chromosomes or other cellular parts have selectively been opened, cut or eliminated without impairing cell viability. Within an entire organism, C. elegans, single cells have been selectively eliminated or fused by distinct laser shots.

The focused laser allows to poke minute holes into cells and nuclear cell walls, which were closed by the cell itself within a few seconds or minutes. This enables injection of, e.g., drugs or genetic material without using viral vectors or chemical treatment of the cells.

The PALM® MicroBeam is the state-of-the-art laser system for non-contact microdissection, pressure catapulting and microsurgery. The method of laser mediated live cell handling promises to take a big step forward in all fields of science related to the study of live cells.

Detection of single cancer cells in patient blood and bone marrow

Siri Tveito. Department of Tumor Biology, The Norwegian Radium Hospital, N-0310 Oslo, Norway (c/o heidi.rasmussen@labmed.uio.no)

The talk will be a presentation of how we at The Norwegian Radium Hospital can select and transfer single cells from a solution and then do RT-PCR. For this kind of applications we use the CellPick system from Nikon/MMI Molecular Machines & Industries AG. We will introduce the system and how it works. Present we are working on detection of single cells in patient blood and bone marrow. We will present our results so far. Also we would like to present some other projects we plan to work on in future by using this automated system for manipulation of living cells in suspension.

Cytogenetic Investigation of Laser-Capture Microdissected Carcinoma In Situ Cells from Testicular Tissue

Anne Marie Ottesen. *Department of Growth and Reproduction, GR-5064, Juliane Marie Centre, University Hospital of Copenhagen, Copenhagen, Denmark* (ANNEMARIERH06096OTTESEN@rh.dk)

A series of cytogenetic investigations of overt testicular germ cell tumours, seminomas and nonseminomas previously revealed a characteristic pattern of chromosomal imbalances. However there were only few studies of carcinoma in situ (CIS), the precursor cell of testicular germ cell tumours of adolescents and young adults. This mainly was due to technical problems caused by a low number of CIS cells located within the testicular tubules surrounded by several other types of germ cells as well as somatic cells. To overcome this problem, we laser-capture microdissected CIS cells from nine cases of CIS, either from tissue without any invasive tumour or from testicular parenchyma adjacent to overt tumours. Prior to detection of cytogenetic abnormalities by high-resolution comparative genomic hybridisation (HR-CGH) analysis, DNA was amplified by degenerate oligonucleotide primed PCR (DOP-PCR) and directly labelled with a mixture of FITC-dUTP and FITC-dCTP. HR-CGH analysis revealed extra chromosome arm 12p material in six out of seven cases with CIS adjacent to overt tumours. These cytogenetical data indicated that extra 12p material is not present in the “ dormant” CIS cell prior to development of an invasive tumour. Thus the gain of extra chromosome 12 material is most likely associated with a more malignant progression of the CIS cell and may not be an early event in the neoplastic transformation. In addition, this study confirmed that laser-capture microdissection followed by DOP-PCR of purified DNA provides good quality material suitable for CGH analysis even in low numbers of scattered cells.