Welcome to the International Symposium on Microgenomics 2014

Dear Participants,

On behalf of the local organizing committee, I want to welcome you to Paris for the 1st International Symposium of Microgenomics. Please take this opportunity to network with your colleagues from around the world and across the scientific spectrum. This meeting is intended to be fully interactive, to build on existing collaborations, and to start new ones.

Around 160 scientists from Europe and the USA will attend this first symposium. We are going to do our utmost to encourage scientific collaboration using every possible means. We will have various social events as part of the symposium that we hope will be a moment for fruitful exchanges between you and will leave you with good souvenirs. On Thursday we will have a Poster session during the Wine & Cheese Reception, and the evening, we will leave the Cordeliers Campus and go to the "Capitaine Fracasse" for the group dinner.

The program of the Microgenomics 2014 symposium covers a wide range of techniques and methods. The ultimate goal of the congress is to provide a comprehensive view of current knowledge to obtain high quality molecules and future developments in "omic" tools (DNA, RNA and protein) for genome analysis and its expression at the cell level. We hope that by the end of the symposium, each participant will be able to choose what methods are the most well-adapted to his or her scientific project.

The scientific and organizing committees of the Microgenomics 2014 Symposium express their warm thanks to the many contributors in the process who made the organization of the congress a pleasant task; to the editing committee and chairpersons, whose expertise was essential to the publication and discussion of the scientific contributions; and to our sponsors whose support is essential for the success of this first congress.

We are sure that all of you will have a productive meeting from a scientific point of view and that you will also enjoy the social events, landscape, culture and hospitality in Paris.

We are very happy to welcome you this spring to Paris, during 2 days of a highly professional, successful and enjoyable event.

Claudia Bevilacqua

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Thursday, May 15 2014

8:15-9:20	**Registration**
9:20-9:30	**Welcome / Introduction (Organizer)**
Session 1	**SAMPLING METHODS**
Chairman:	M. DALOD
9:30-10:15	Keynote lecturer: M. DALOD (France) Characterization of rare immune cell types through gene expression profiling
10:15-10:45	Invited lecturer: H. LUCHE (France) DeCIPHEr critical steps in a flow cytometry experiment to ensure reproducible and high quality datasets
10:45-11:15	Coffee break • Posters • Sponsors
11:15-11:45	Invited lecturer: B. LISS (Germany): Correlating function and gene expression at the level of individual dopamine neurons - from mice to men
11:45-11:55	Selected oral presentation T. HEAMS (France): Microgenomics and Epistemology
11h55-12:05	Selected oral presentation O. BERTHUY (France): Cell encapsulation for multiplex culture on a chip
Session 2	**MICROGENOMICS AND DNA**
Chairman:	C. KLEIN
12:05-12:50	Keynote Lecturer: C. KLEIN (Germany) Impact for therapy selection and the understanding of cancer evolution
12:50-13:00	Selected oral presentation E. ROSSI (Italy): Are apoptotic Circulating Tumor Cells (CTCs) suitable for molecular analysis?
13h00-14:30	**Lunch • Posters • Sponsors**
14:30-15:00	Invited lecturer: P. PINZANI (Italy): Circulating tumor cells: from enrichment to single cell sequencing
15:00-15:10	Selected oral presentation P. PATERLINI (France): Single cell genetic analysis helps validating cytopathological identification of CTCs in patients with Clear Cells Renal Carcinoma
Session 3	**MICROGENOMICS AND RNA: MIQE guideline and microgenomics**
Chairman:	M. KUBISTA
15:10-15:55	Keynote lecturer: M. KUBISTA (Sweden) Taking Expression Profiling to New Dimensions
15:55-16:05	D. FERRARO (France) Droplet microfluidics for gene expression study at the single cell level
16:05 -16h35	**Coffee break • Posters • Sponsors**
16:35-17:05	Invited lecturer: K. LIVAK (USA) Driving Genomics to the Single-Cell Level: Analysis of RNA Expression Using qPCR and RNAseq
17:05-17:35	Invited lecturer: S. JACKSON (USA) Gene expression profiling from Single Laser Capture Microdissected (LCM) Cells
17:35-17:45	Selected oral presentation: J. BELLIERE (France) Single-cell transcriptional analysis of FACS-sorted kidney macrophages
17:45-17:55	A. Eugster (Germany) Gene expression of autoreactive T cells - Biomarker and pathogenesis of Type 1 diabetes
17:55-19:00	Poster session + French Wines & Cheeses
20:00-22:30	Dinner cruise on the Seine

Friday, May 16 2014

Session 3 MICROGENOMICS AND RNA: MICRORNA AND NGS

Chairman: M. PFAFFL

9:00-9:45	Keynote lecturer: M. PFAFFL (Germany) Exosome isolation and holistic expression profiling using RNA-Seq and RT-qPCR
9:45-10:15	Invited lecturer: K. HOEFIG (Germany) Measuring microRNA expression in
10:15-10:25	Oral presentation: H. AUER (Spain) Towards a Comprehensive Single Cell Expression Profiling
10:25-10-35	Selected oral presentation B. LANGELIER (France): A combination of microgenomics approaches to understand the impact of gut microbiota on the regulation of the hypothalamo-pituitary-adrenal axis in rats
10:35-11:15	Coffee break • Posters • Sponsors
11:15-11:45	Invited lecturer: R. SATIJA (USA) Comparative analysis of RNA sequencing methods for degraded or low-input samples
11:45-11:55	Selected oral presentation: B. DUBREUCQ (France) Low RNAseq microdissected plant tissue
11:55-12:10	GOLD SPONSOR presentation: S. Jackson (USA) Genomic Characterization of Challenging or Limited Research Samples using the Ion AmpliSeq™ Targeted Re-Sequencing Technology
12:10-14:00	Lunch

Session 4 MICROGRENOMICS AND PROTEINS

Chairman: L. LIOTTA

14:00-14:45	Keynote lecturer: L. LIOTTA (USA) Advantages or disadvantages of different methods for proteomics analysis
14:45-14:55	Selected oral presentation: R. DALBIES-TRAN (France) Oocytomics: combining transcriptomics and proteomics to understand post-transcriptional regulation in bovine oocyte
14:55-15:05	Selected oral presentation: C. TARAGNAT (France) Combining surface plasmon resonance and mass spectrometry to identify Bone Morphogenetic Protein (BMPs) interactant
15:05-15:35	Coffee break • Posters • Sponsors
15:35-16:05	Invited lecturer: M. WISZTORSKI (France) Mass Spectrometry Imaging coupled to Microproteomics: From imaging to identification of proteins on tissue section
16:05-16:33	Invited lecturer: D.W. ROSENBERG (USA) Unlocking the prognostic significance of microdissected proximal lesions from human colon
16:35-16:45	Closing remarks

A copy of the abstract book is available on the flash drive provided in the symposium bag

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ORAL PRESENTATIONS

SESSION 1: SAMPLING METHODS

Chairman: Marc Dalod

Characterization of rare immune cell types through gene expression profiling

DALOD M[1]

The mammalian immune system encompasses a variety of cell types endowed with specific, complementary, functions. The definition of what are distinct cell types as opposed to what are different developmental or activation states of a given cell type is not always easy to make. Only very few cell surface molecules are each expressed in a specific manner on a given immune cell type under steady state conditions. This is even worse under activation conditions. Therefore, the use of a small number of cell surface markers as previously done by "oligoparameter" flow cytometry approaches can sometimes be deceiving and lead to erroneous interpretations regarding cell type identity. Moreover, the markers used to define cell types can considerably vary depending on the mammalian species studied, including between mice and humans. Studying ontogeny to define cell type identity is considered as the gold standard approach in mice but is extremely difficult to do in animal species not amenable to genetic manipulation for in vivo inactivation of transcription factors or for cell-fate mapping experiments. Functional studies are the most informative approaches to understand cell type identity and the most relevant for translational purposes, however such studies require prior knowledge of the potential functional specialization of the cells to select the adequate functional tests to perform, are often costly, time-consuming and less well standardized. With the development of high throughput approaches for characterizing the gene expression programs of cells even when starting with very low amounts of input material, it has become possible to seek for a less subjective, more rigorous, potentially unbiased definition of cell types based on expression of complex transcriptomic signatures of tens or hundreds of genes. The analysis of these transcriptomic signatures will then help generate new hypotheses on the ontogeny and functions of cell types that can be tested experimentally. We and others demonstrated the power of transcriptomics for solving the identity of cell types harboring an ambiguous cell surface phenotype (1-5) and for translating knowledge on immune cell subset ontogeny and functions from mice to humans or other species (1, 6-9). However, performing gene expression profiling of a given immune cell type is generally achieved after purifying this population using the conventional, biased approaches relying on its phenotypic definition by the combination of a small number of cell surface markers. In other words, in the initial phases of gene expression profiling studies of immune cell types, the approach is still limited by the biases linked to the classical phenotypic definition of immune cells. Hence, during the corresponding sampling procedures, critical steps must be followed to decrease a priori the risk of contamination by another cell type or even of erroneous definition of the cell identity. Additional control steps are required at the time of analysis of the transcriptomics data to re-evaluate these risks a posteriori. These steps will be described through concrete illustrations and discussed with regards to different sampling methods available to purify immune cell types, discussing their respective advantages and drawbacks as well as their complementarity. Once first series of gene expression profiling have been obtained, this helps refining the phenotypic definitions of cell types by identifying combinations of cell surface markers more specific for each cell type and eventually conserved across tissues, activation conditions and species (1, 6-14). If required, this can then allow generating even more robust transcriptomics data for cell types that had not yet been defined with sufficient rigor. Finally, we will discuss the advances that are being brought in this research field through single cell transcriptomic analyses as recently illustrated by other research teams (15).

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DeCIPHEr critical steps in a flow cytometry experiment to ensure reproducible and high quality microgenomic dataset

LUCHE H[1], HADJEM L[1], MELLO M[1], GRENOT P[1], MALISSEN B[1,2,3,4], MALISSEN M[1,2,3,4]

Recent years have witnessed the growth of functional genomics projects focusing on the use of the laboratory mouse as a model of human disease. The single gene approach has lasted, and we are now entering a new phase where scientists will think in terms of networks and pathways. The Centre for ImmunoPHEnomics - CIPHE (INSERM/US012, AMU, CNRS/UMS3367) is a new institute dedicated to phenogenomics studies. With its cutting-edge expertise in mouse genetics and immunology, the CIPHE aims to develop and analyze in a massively parallel and standardized mode mouse KO/KI models allowing the understanding of the function of the mouse immune system under normal and infectious conditions.

The main investigation technique of the CIPHE immune-phenotyping module is cytometry. By combining our expertise in flow cytometry and knowledge in immunology, we establish high content immunophenotyping panels (>14C) to investigate all cell subsets of the hematopoietic lineage in the mouse. Thanks to the release of a broad range of bright new reagents and the availability of high-end instruments able to monitor up to 18 fluorescent parameters at the single cell level, HC-MFC could emerge and allow the entry into a cytomic era. The Immgen experience and studies we performed in collaboration with CIML demonstrate that high content multiparameter flow cytometry (HC-MFC) was instrumental in transcriptomic studies on minute populations of highly purified cells. However, setting up HC-MFC experiments is a real technical challenge and each experimental step needs to be carefully examined and controlled to ensure meaningful analysis. In light of our contributive work to the Immgen2 consortium, we will go through all the critical steps in a flow cytometry and cell sorting experiment that need to be considered to ensure reproducible and high quality microgenomic datasets.

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Correlating function and gene expression at the level of individual dopamine neurons - form mice to men

LISS B[1]

The dopamine midbrain system and the activity of dopamine releasing (DA) neurons are not only essential for motor control, but are also crucial for emotion, reward, and for cognitive brain functions. Accordingly, one can distinguish distinct types of DA midbrain neurons - with distinct axonal projections, and a distinct activity pattern - that are differentially affected in diseases: Substantia nigra (SN) DA neurons, projecting to the dorsal striatum, are particularly prone to degeneration in Parkinson disease (PD). In contrast, neighboring DA neurons in the ventral tegmental area (VTA), projecting to limbic as well as cortical areas, are significantly less vulnerable to degeneration in PD, but are particularly affected in Schizophrenia and attention deficit hyperactivity disorder (ADHD). The cause for this selective vulnerability of DA midbrain neurons to distinct diseases is still unclear. However, understanding the molecular mechanisms of distinct SN DA and VTA DA function and signaling in health and diseases is the prerequisite for the development of novel, cell-specific therapies. Thus, our main research goal is to identify cell-specific functional and molecular differences of SN DA and VTA DA neurons, defining their distinct physiological functions and their selective transitions to disease states. By combining *in vivo* retrograde tracing of distinct DA projections with *in vivo* and *in vitro* electrophysiology as well as UV-lasermicrodissection, and quantitative PCR-based gene-expression profiling at the single cell level, we are defining molecular signaling-pathways that control cell-specific DA neuron activity patterns, as well as selective disease pathways, in particular in PD. We correlate quantitative electrophysiological function with cell-specific gene-expression of individual SN DA and VTA DA neurons from respective mouse-models, and we transfer our mouse model finding to human disease by analyzing DNA and RNA in DA neurons from human post mortem brains (e.g. PD vs controls). We developed a specific RT-qPCR approach that allows parallel mRNA and miRNA quantification in individual neurons, and that also takes the important issue of variable RNA integrities and nonoptimally matched human samples into account. Non-optimally matched donor ages and RNA integrities (RIN) are common problems when analyzing human samples. We addressed and dissected the influence of distinct ages and RIN of our human SN DA samples by applying a specifically-optimized, linear-mixed-effects model to our quantitative RT- qPCR data (Gründemann et al, MMB 2011; Schlaudraff et al, NBA 2014).We have focused on dissecting the roles of ion-channels and receptors for selective functions and vulnerabilities of DA neurons, since their cell-specific activity directly defines neuronal activity pattern and dopamine release in health and disease states (Lammel et al. Neuron 2008; Schiemann et al. Nature Neurosci. 2012; Dragicevic et al, Brain 2014).

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Microgenomics and Epistemology

HEAMS T[1,2]

By unraveling unexpected amounts of cellular diversity, microgenomics challenges some theoretical pilars of experimental biology. For decades, cellular diversity in multicellular organisms has been explained within a conceptual deterministic framework, explaining the high level of homogeneity within cell types, as well as differences between them. Here, reproducibility is achieved by precise regulation systems, and variation stems from different uses of the same genomic information, mediated by different signals. This successful view encompasses a large number of observations but also faces severe epistemic hurdles. First, it mostly explains the origins of variations by a *reductio ad infinitum*: variations come from previous external variations, resulting in order originating from previous order. Second, it relies on a puzzling evolutionary dichotomy opposing competitive interactions among unicellular organisms, and cooperation between cells of multicellular organisms. In addition, single-cell studies and microgenomics have shown that, even within tissues, individual cells display various unpredictable behaviors, spanning from background noise to actual biological parameters (Kaern et al, 2005). These techniques have also described an extensive range of variations in genome sequence among supposedly clonal cells. Thus, experimental facts not only enrich our view of multicellular dynamics, but also question the very bases which we usually rely on to infer biological explanations. By taking advantage of variations over average values, microgenomics has a key role in addressing some major aspects of this ongoing debate. It could quantify how much probabilistic cellular dynamics can coexist with deterministic ones, and could disentangle genomic, transcriptomic and proteomic origins of phenotypical variations. In doing so, it could document to what extent elementary disorders can contribute to biological order (Kupiec, 2009).

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Cell encapsulation for multiplex culture on a chip

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Introduction

To unravel cell complexity, living-cell chips have been developed that allow delivery of experimental stimuli and measurement of the resulting cellular responses. We have been developing a new concept for multiplexed detection of biomolecules secreted by different cancer cells. In the present report, we show proof of the concept of LNCaP small population spotting for prostate cancer cells on a gold surface (from 1 to 100 cells).

Material and Methods

Antibodies and different cell lines were spotted using a piezoelectric spotter (sciFLEXARRAYER S1, SCIENION, Germany).

Results and Discussion

In order to keep the cells in a hydrated media during the robotized micropipetting and to address different cell lines on a single chip, a biocompatible alginate polymer was used. This approach enables the encapsulation of the cell in a very small volume (50 nL), directly on the substrate and permits a precise control of the number of cells in each alginate bead. After 24h culture, the adherent cells are ready for SPRi experimentation (Figure 1).

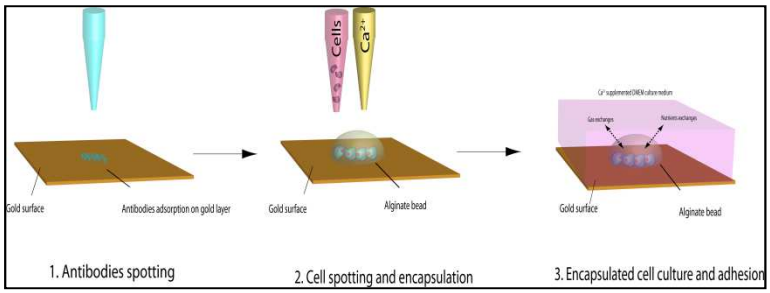

Figure 1. Overview of the microfabrication process

In the final concept, SPRi is used to detect protein secretion (PSA) in response to specific stimuli transported by a microfluidic network. This technique enables real time label-free detection.

Conclusion

LNCaP cells are used as a model system for the characterization of the SPRi cell-chip performances and potentialities.

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Session 2: Microgenomics and DNA

Chairman: Christoph Klein

Molecular profiling of single circulating tumor cells: Impact for therapy selection and the understanding of cancer evolution

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Introduction

About 230 clinical trials currently explore the role of circulating tumor cell (CTC) analysis for therapy decisions, but no assays enable comprehensive molecular characterization of CTC with diagnostic precision.

Material and Methods

We therefore combined a workflow for CTC enrichment and isolation with 100% purity with a non-random whole genome amplification method for single cells and applied it to 510 single CTC and 189 leukocytes of 66 breast cancer patients.

Results and Discussion

We defined a genome integrity index (GII) to identify cells suited for molecular characterization by different molecular assays in more than 90% of single cells, such as diagnostic profiling of point mutations, gene amplifications and whole genomes of single cells. The high reliability on clinical samples enabled assessing the molecular heterogeneity of single CTC of metastatic breast cancer patients. We readily identified therapeutically relevant genomic disparity between primary tumours and CTC.

Conclusions

Microheterogeneity analysis among individual CTC uncovered preexisting cells resistant to *ERBB2*-targeted therapies suggesting ongoing microevolution at late stage disease whose exploration may provide essential information for personalized treatment decisions.

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Are apoptotic Circulating Tumor Cells (CTC) suitable for molecular analysis?

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Introduction

CTC represent a "liquid biopsy" that can be used to tailor treatment for individual patients. However, the main requirement for the use of CTC as a real time liquid biopsy is that CTC condition is sufficient to detect the presence of treatment targets. Frequently, even in the early stages of malignancies (Rossi et al., 2010) there are very few CTC and a high percentage of apoptotic cells. We decided to assess whether the DNA of these cells is satisfactory for further molecular analysis.

Material and Methods

CTC preparation were obtained by CellSearch assay (Cristofanilli et al., 2004). To obtain single-cell' samples, we used either the FACS sorting or laser microdissection. We collected the single-cell' samples at different stages of apoptosis as determined by specific markers, namely M30, yH2AX, Caspase 3, Annexin V/PI.

Results and Discussion

We treated breast-cancer cell lines (SKBR3 and MCF-7) with different drugs (cisplatin, taxol, etc.) to induce apoptosis in these cells. We spiked these cells into the peripheral blood of healthy donors, at concentrations reported in *ex vivo* samples of cancer patients. Then, we processed the spiked samples to obtain single-cell samples; the individual cells positive for a single marker of apoptosis or for two markers (early or late phase of apoptosis) were processed with the WGA protocol. In parallel, apoptotic cells were isolated from a patient by laser micro-dissection and processed with the WGA protocol. The concentrations of all samples (cell lines and patient) were sufficient both for classic sequencing methods and for NGS library preparation.

Conclusions

Despite the large quote of apoptotic cells, CTC are suitable for further molecular analysis. Accrual of patients is ongoing; updated data will be available and presented at the meeting.

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Gene Expression In Stromal And Epithelial Laser-Microdissected Prostate Cancer Tissues

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Introduction

Prostate cancer is one the most frequent causes of death from cancer in men in western countries. At present there are no prognostic markers of prostate cancer progression. Metastatic patients are treated with androgen deprivation therapy with scarce efficacy because the disease becomes rapidly androgen-independent. Androgen receptor (AR) is known to be involved in initiation and development of prostate cancer and recently it has been shown to play a different role in the epithelial and stromal compartments of the tumor, acting as tumor suppressor in the former and inducing disease progression in the latter. In this study we aimed at optimizing a protocol for studying gene expression of AR and other markers of tumor progression, such as PSA, EGFR and PTEN, in stromal and epithelial compartments of prostate cancer.

Material and Methods

AR, EGFR, PSA and PTEN mRNA expression was analyzed by qPCR in frozen prostate cancer specimens from 20 patients after laser microdissection of the stromal and epithelial compartments. RPL13a (coding for a ribosomal protein) was chosen as the reference gene. cDNA from samples was preamplified by Taqman Preamp Master Mix (Life Technologies). Preamplification Uniformity was checked on a control sample obtained by extracting RNA from the prostate cancer cell line LNCaP.

Results and Discussion

We successfully set up a preamplification protocol of cDNA involving 20 cycles of preamplification. PSA was more expressed in the epithelial than in the stromal compartment of the tumor, while PTEN presented a higher expression in the stroma. A significant correlation was found between AR and EGFR expression in the epithelial compartment.

Conclusions

Tumor stroma supports prostate cancer progression and several studies show that it may be a promising therapeutic target, therefore it is necessary to optimize methods for investigating the tumor microenvironment. In this context our research provides a contribution demonstrating the possibility of studying gene expression on very limited amounts of input materials such as laser- microdissected prostate cancer tissues.

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Single cell genetic analysis helps validating cytopathological identification of CTC in patients with Clear Cells Renal Carcinoma

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Introduction

Cytopathological identification of circulating tumor cells (CTC) is a critical issue in non-invasive predictive oncology. However, in very rare cases, such as kidney cancers, the diagnostic value of cytology is weak. We thus planned to study the impact of genetic molecular analysis in combination with cytology in the CTC field. To this aim, we have taken advantage from the model of Clear Cell Renal Carcinoma (CCRC), which is characterized by the VHL genetic marker, and used genetic analysis in addition to the cytopathological study of CTC.

Materials and Methods

We treated by ISET the blood of 30 patients with CCRC, collected before surgery, to isolate rare circulating cells. Cytopathological analysis was performed blindly by 3 pathologists on the isolated cells to identify CTC and distinguish them from circulating cells with uncertain malignant features (CC-UMF) and from cells with benign features (CC-BF). CTC, CC-UMF and CC-BF were then individually microdissected. Single cell DNA was preamplified, amplified by VHL-specific primers and analyzed by sequencing. VHL-specific genetic analysis was performed blindly in microdissected cells and in the corresponding tumorous tissue.

Results and Discussion

We found CTC/ CC-UMF in 29/30 analyzed patients with CCRC. VHL mutations were found in the tumor of 25 out of the corresponding 29 CCRC tumors. Among 327 microdissected CTC/ CC-UMF, we obtained VHL-specific results in 205 including 64 CTC and 141 CC-UMF, according to the cytopathological analysis. VHL mutations were blindly detected in 57/64 CTC and in 125/141 CC-UMF. These results were analyzed according to the presence or absence of VHL mutations in the tumor tissue.

Conclusion

This is the first study using single cell genetic analysis and cytopathology to study CTC in patients with CRCC. Our results show that genetic analysis is a very interesting approach potentially complementing cytopathology for the diagnosis of CTC.

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SESSION 3(a). MICROGENOMICS AND RNA: MIQE GUIDELINE AND MICROGENOMICS

Chairman: Mikael Kubista

High throughput single cell expression profiling

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Introduction

Biological samples are complex, being composed of large number of cells of different types. When studying traditional samples containing many cells only the collective response of all the cells present is measured. However, the cells may respond differently and a small subpopulation may be critical. Today, these systems can be studied using single cell expression profiling. Here we applied single cell profiling to study the response of astrocytes to brain trauma using a mouse model. We also studied asymmetric cell division during early development of *Xenopus laevis* by single cell and intracellular profiling using qPCR tomography.

Material and Methods

Single astrocytes from mice were enriched and collected by FACS using GFP under the control of the GFAP promoter as marker. Cells were lysed (Cellulyser, TATAA Biocenter), reverse transcribed (GrandScript, TATAA Biocenter), pre-amplified (GrandMaster PreAmp, TATAA Biocenter), and profiled using high throughput microfluidic qPCR (BioMark, Fluidigm). Data were pre-processed and cells were classified using multivariate methods (PCA, SOM, clustering) and correlation analysis with the GenEx software (ver. 6, MultiD Analysis).

Results and Discussion

Astrocytes were collected from mouse brains at different time points after the induction of focal ischemia. Each cell was profiled for the expression of 47 genes. Classification revealed astrocyte reactivation with the formation of distinct subtypes (Figure). Single cell and subcellular blastomere profiling revealed asymmetric cell division is induced by asymmetric distribution of key cell fate determinants already in the fertilized cell.

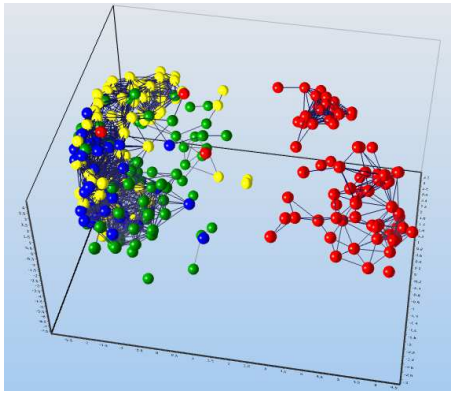

Figure 1. Dynamic PCA classification of reactive astrocytes at 0 (blue), 3 (yellow), 7 (green) and 14 (red) days after brain trauma. Classification with GenEx (MultiD)

Conclusions

Single cell profiling is most powerful to study complex biological samples, revealing heterogeneity and to identify key expression pathways active in critical cell types. Power and robust flows for experimental and analytical analysis are available, as well as highly optimized reagents.

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Droplet microfluidics for gene expression study at single cell level

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Introduction

Investigating the mutated status of genes in tumor cells and how altered expression of genetic variants contributes to their development are key issues in the understanding of cancer. Standard laboratory methods are still not adapted to the isolation and quantitation of low mRNA amounts and new techniques need to be developed in particular for rare subset analysis. By reducing the volume involved, time process and the contamination risks, droplet microfluidics provide numerous advantages to perform analysis down to the single cell level[1]. We present here an innovative approach for the mRNA extraction and transcription at the single cell level, based on combination of droplet microfluidic and magnetic tweezers technology[2]. This technology has been validated in terms of sensitivity and robustness with the β-actin and HER2 transcripts.

Material and Methods

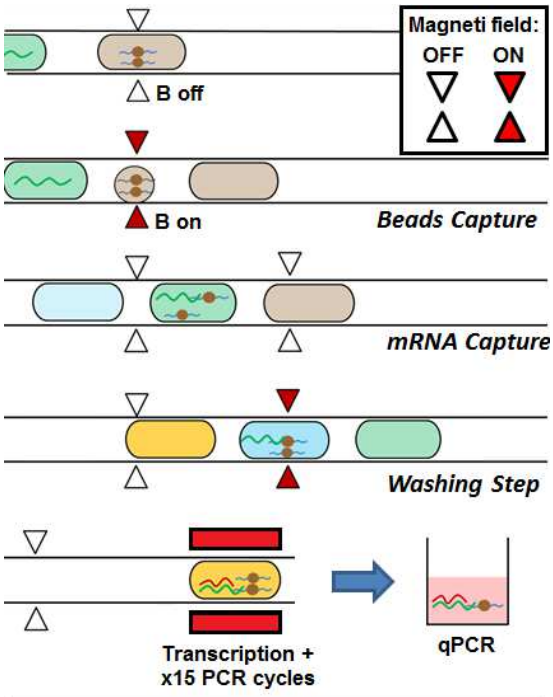
mRNA purification kit based on oligo-dT beads coated was integrated in 200nL droplets to capture mRNA from total RNA. The whole analytical workflow was integrated in droplets (mRNA extraction, transcription and PCR). Final qPCR was done off-chip (Smart Cycler, Cepheid). (Figure 1).

Results and Discussion

Using this technology we were able to detect both β–Actin and HER2 genes at the single cell level. The technique was observed to be extremely stable and reproducible.

Conclusions

Compare with the standard procedure, this technique allows reducing the volume by 100 times, the contamination risks and taking advantage of the high throughput droplet production. In a near future, cell lysis will be integrated in the droplet approach to integrate a complete single cell analysis while other genes of interest in cancer will be investigated.



	Total RNA (pg)			
	200	20	2	0,2
Ct (β-Actin)	15,1	18,7	22,0	27,0
Ct (HER2)	23,4	25,8	30,4	

Figure 1: (Top) experiment workflow; (Bottom) qPCR results.

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Driving Genomics to the Single-Cell Level: Analysis of RNA Expression Using qPCR and RNA Seq

LIVAK, KJ[1]

Introduction

The stochastic nature of generating eukaryotic transcripts challenges conventional methods for obtaining and analyzing RNA expression data. The basic problem is how to deal with noisy data. The use of microfluidics makes it cost effective to look at a sufficient number of genes and a sufficient number of single cells to help address this noise issue.

Materials and Methods

Single cells were collected either by FACS into 96-well plates or using the Fluidigm® C_1™ Single-Cell Auto Prep System. qPCR was performed using 96.96 Dynamic Array™ IFCs in the Fluidigm BioMark™ HD System. For mRNA seq, libraries were sequenced using 100-bp paired end reads on an Illumina® HiSeq 2500, generating approximately four million reads for each C_1 single-cell library.

Results and Discussion

In an eQTL study, qPCR quantification of 92 transcripts affected by Wnt signaling in 1,440 single cells from 15 individuals identified 47 significant associations between SNP and gene expression phenotypes. This compares to finding only six SNP associations if mean expression, rather than single-cell expression, is used. This illustrates the power of using single-cell analysis to enhance the resolution of genotype-phenotype correlations. In a study of the differentiation of primary human myoblasts, a new unsupervised algorithm called Monocle was developed to analyze single-cell mRNA seq data. This algorithm can resolve the asynchrony present in a population of differentiating cells and order single cells along a developmental trajectory.

Conclusions

Despite the noise inherent in single-cell transcriptomic data, analysis methods are being developed that can extract meaningful biological insights.

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Gene Expression Profiling from Single Laser Capture Microdissected (LCM) Cells

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Introduction

Gene expression profiles have traditionally been generated by analyzing the average expression in a tissue or group of cells. However, the average expression may not be a true representation of the different profiles that could exist even in a pure cell population (e.g., in different states of growth, differentiation, or activation). As a result, the transcriptional variability of individual cells and any insight into the relationship between specific genes in single cells are lost. To fully understand the complexity of tissue and cellular heterogeneity, it is therefore necessary to measure molecular signatures at the single cell resolution. Laser Capture Microdissection (LCM) is a technique that provides a rapid and reliable method to select purified cell populations. Using LCM, single cells or groups of cells from a heterogeneous tissue sample, can be isolated. This allows a targeted approach to genomic profiling. In this study, we developed a workflow for gene expression profiling of single cells dissected by LCM.

Material and Methods

The Arcturus^{XT}™ LCM system was used to harvest single cells or small groups of cells from frozen human tumor tissue samples. RNA was extracted and then analyzed for quantitative RT-PCR (qRT-PCR) gene expression using the Single Cell Pre-Amp mix and the TaqMan® OpenArray® Human Cancer Assay on a QuantStudio™ 12K Flex.

Results and Discussion

We show that individual cells do indeed have very different expression profiles; however, combining the profile from many cells approaches the pattern seen in the average profile.

Conclusions

These results demonstrate that LCM, coupled with a high-throughput expression profiling platform, can facilitate screening of a large number of genes from single cells isolated from tissue sections.

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Single-cell transcriptional analysis of FACS-sorted kidney macrophages

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Introduction

Acute kidney injury (AKI) is a life-threatening complication of rhabdomyolysis. Macrophage infiltration has been observed in kidney after rhabdomyolysis [1], but its role is unclear. Macrophages exhibit diverse phenotypes [2]: whereas M1 are pro-inflammatory, M2 are associated with repair [3]. Previous characterization of kidney macrophages at "population" level suggests the following: (i) either the coexistence of M1 and M2 cells, (ii) or that macrophages simultaneously express both M1 and M2 markers. To address this question, we analyzed kidney macrophage gene expression at the "single-cell" level.

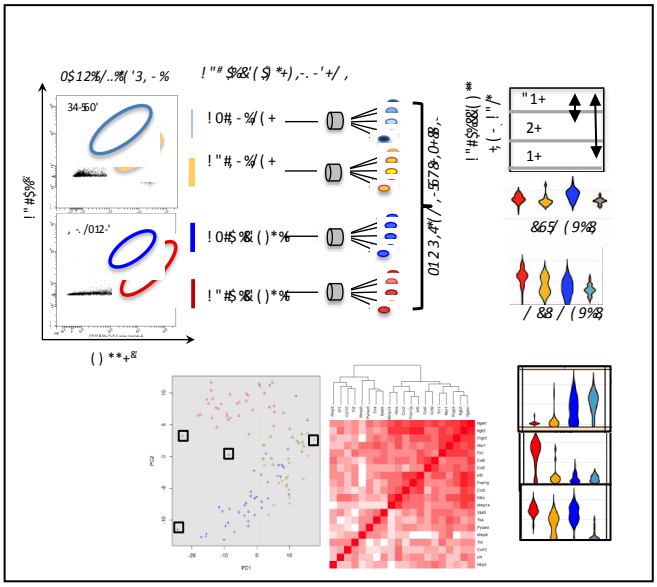

Figure 1. Study design and examples of bioinformatics analyses.

Materials and Methods

Rhabdomyolysis was performed in mice through intramuscular Glycerol (Gly) injection or saline as control. Macrophages were isolated from digested kidneys, and FACS-sorted into 2 sub-types (R1 and R2) according to the expression of membrane markers (F4/80 and Cd11b). Sorted cells were loaded into C1™ Single-Cell-Auto-Prep-System. Amplicons were used on qPCR Biomark®. In total, 104 cells and 89 genes were analyzed.

Results and Discussion

Most of the genes exhibited heterogeneous expression within cell populations. Some genes were up-regulated in the glycerol-condition irrespective of the R1/R2 status. Positive correlations between genes were identified, indicating co-expression. Non-supervised hierarchical clustering and principal component analysis clearly discriminated R1-Gly from R2-Gly. FACS-sorted populations were heterogeneous and individual cells simultaneously expressed both M1 and M2 markers. These data suggest that R1-Gly cells had a potential to become M2, and that R2-Gly cells could exert anti-fibrotic activity. Four targets were verified as macrophage-dependent *in situ*.

Conclusions

Despite their common identity defined by cell sorting, we provided evidence for different cell state transitions inside kidney macrophages infiltrate during rhabdomyolysis-induced AKI.

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Gene expression of autoreactive T cells - Biomarker and Pathogenesis of Type 1 diabetes

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Introduction

Islet autoantibody seroconversion is the first detectable evidence of ongoing autoimmunity in the process leading to Type 1 Diabetes. While autoantibodies are markers of pre-diabetes, autoreactive T cells specific for beta-cell autoantigens are key to beta cell destruction. The BABYDIET study (1) followed children with a strong genetic predisposition for type 1 diabetes during infancy and offered the possibility to search for T cell activation against islet antigens. We used samples from this study to identify the chronical appearance of beta cell autoantigen primed $CD4^+$ T cells and characterised their phenotype by analyzing gene expression profiles.

Materials and Methods

PBMC: pre- and post seroconversion from auto-Ab negative or positive children. CFSE labeling and culturing with or without antigen for 5 days. Antigen responding $CD4^+$ memory T cells are single cell sorted. Analysis of cytokine profiles by RT-PCR on the Fluidigm 96.96 dynamic array.

Results and Discussion

We find that primed T cell responses are specific to autoantibody positive children and appear predominantly around the age of seroconversion. Comparing gene expression profiles of autoantibody positive and negative children we see that reactive cells differ already prior to seroconversion, and that they may predict the risk of autoimmunity. Evidence of regulatory responses is found in autoantibody negative – but not positive children. After seroconversion, autoantigen reactive cells develop an $IFNg^+$ profile, differing from a vaccine responder profile.

Conclusions

Gene expression profiling of antigen responsive T cells provides both biomarkers and pathogenesis discovery in Type 1 Diabetes.

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Session 3(b): Microgenomics and RNA: microRNA and NGS

Chairman: Michael Pfaffl

Exosome isolation and holistic expression profiling using RNA-Seq and RT-qPCR

PFAFFL[1] MW AND KIRCHNER B

Introduction

Small RNA, in particular microRNA, regulate gene expression by post transcriptional binding and thereby suppressing protein translation. They are present in most eukaryotic cells and play an important role in almost all physiological or regulative processes. Small RNA were detected in various matrices, such as blood, plasma, saliva and urine. However, very less information is available about the small RNA composition in biofluids such as milk and whether milk possesses its own defined small RNA profile differing from blood. Furthermore the small RNA transcriptome differences between whole milk versus milk exosomal isolates were investigated.

Materials and Methods

To generate a holistic overview of all present small RNA Next Generation Sequencing (small RNA-Seq) was performed on whole blood, whole milk and exosomes during the first lactation days. Exosomes were purified via ultracentrifugation, due to the higher exosomal and RNA quality. Small RNA-Seq was performed using an Illumina HiSeq 2500 and subsequent data analysis was done independently, using either the GGS and GGA stations from Genomatix Software GmbH (Munich, Germany) or using freely available python scripts and R-packages (Bioconductor). First focus was on the dynamic regulation of microRNA in milk and/or exosomes in comparison to blood. Significant regulation of microRNA between different tissues and time points was defined by a fold change of expression of at least 2-fold and a Benjamini-Hochberg adjusted p-value of less than 0.05. To validate these findings key microRNA were quantified via RT-qPCR for fold change comparisons. Experimentally validated mRNA targets for regulated microRNA were taken from the Tarbase 6.0 database from Diana Lab (Athens, Greece) and pathway analyses were generated using GePS (Genomatix Pathway System).

Results and Discussion

RNA Seq clearly showed that milk and exosomes possess its own unique small RNA profile compared to blood. This highlights its dynamic changes during the first lactation days. Pathway analysis for affected targets revealed significant impact on cell cycle progression, cell adhesion, DNA repair, apoptosis, and oncogenic defense.

Conclusion

This study underlines the potential role of microRNA (and small RNA in general) in mammary gland physiology. Milk microRNA and exosomes seem essential for the mammary gland immune system, but also as an active modulator in the newborn calf.

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Measuring MicroRNA Expression in Size-Limited Samples

HOEFIG K[1] AND HEISSMEYER V

Introduction

MicroRNA are small non-coding RNA of an average length of 22 nucleotides, which repress translation of a large number of target mRNA. The particular importance of this group of small RNA arises from the ever growing evidence that they control many biological processes and that deregulation of individual microRNA frequently results in cancer. The expression of microRNA is spatially and temporarily fine-tuned and expression levels can reach more than 50 000 copies of one microRNA within a single cell. It is well documented that the comparison of microRNA signatures of normal and diseased tissues results in a small number of differentially expressed microRNA, which are consequently of high diagnostic value. However, measuring microRNA expression can easily produce false-positive results, due to the high sequence similarity of the microRNA within families and because biologically inactive pre-microRNA as well as contaminating bystander cells may falsify the signal.

Material and Methods

The application of a quantitative PCR-based method is described here to specifically and reliably detect microRNA expression levels from as little as 50 cells. Pure cell populations were either derived from fluorescence activated cell sorting (FACS) or from laser capture microdissection (LCM). Additionally, a combination of quantitative PCR and LCM can be applied to measure microRNA expression of cells obtained from formalin-fixed, paraffin-embedded tissues.

Results and Discussion

We measured miR-155 expression of FACS-sorted germinal center T cells, derived from an immunized mouse, and compared it to mixed splenic CD4+ T cells, obtained from a non-immunized mouse. The endogenous miR-155 expression was approximately six times higher in germinal center T cells than in a mixed CD4+ T cell population. In a second approach it was demonstrated that LCM can be used to isolate cells that cannot be accessed by FACS.

Conclusions

We established simple FACS and LCM-based methods to access rare cell samples of normal and diseased tissues for miRNA-profiling.

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Towards a Comprehensive Single Cell Expression Profiling

PONS XIMENEZ JI[1], RODOLOSSE A[1], SCHWEITZER A[2], VASSENA R[3], AUER H[1].

Introduction

Quality of expression profiling methods from thousands to millions of cells is well established, validated and characterized in its reproducibility (Shi *et al.*, 2006). In contrast, methods for profiling small cell populations are published since over 20 years but generally lack validation of reliability of transcript measurements.

Material and Methods

Here we present experience with and improvements of Pico Profiling (Gonzalez-Roca *et al.*, 2010), a workflow of RNA isolation and cDNA amplification for cell populations as small as a few cells or even a single cell.

Results and Discussion

The cDNA generate by Pico Profiling is suitable for analysis by qPCR, microarrays or sequencing. We show that these measurements from extremely small populations are as informative as standard techniques applied to thousands or millions of cells. Expression profiles from cDNA generated by Pico Profiling are highly comparable to those produced by standard protocols of Real-time PCR or microarray analysis. Latest improvements include quantification of long non-coding RNAs and alternatively spliced transcripts. Some examples from a long line of biological projects will be presented, in which Pico Profiling provided biological conclusions that could not have been drawn with alternative methods.

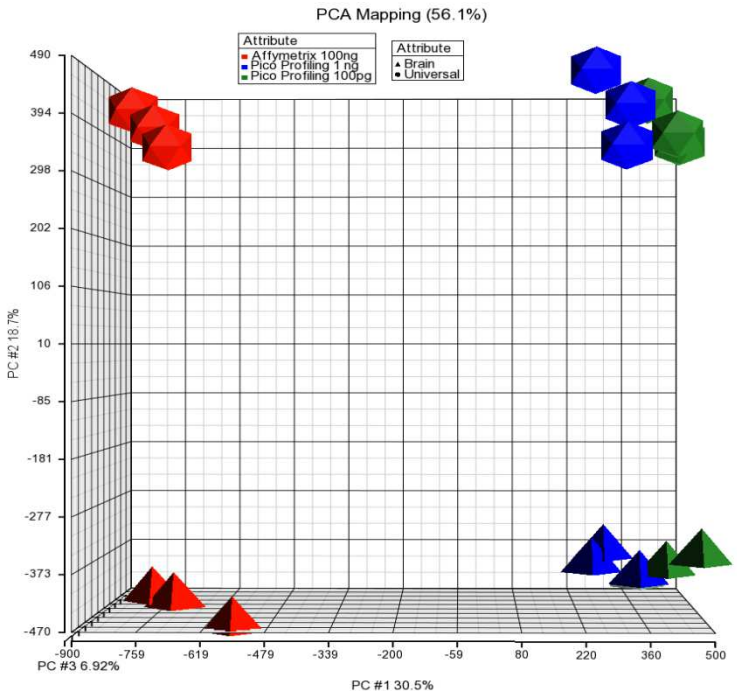

Figure 2 (Principle Component Analysis of MAQC samples A and B (URR, icosahedrons and brain, pyramids respectively), analyzed using standard protocol (100 ng RNA, red), and Pico Profiling (1 ng RNA, blue, 100 pg, green). Expression values were summarized on the exon level.

Conclusions

Pico Profiling, as presented here, allows generating accurate expression profiles from cell populations as small as ten cells.

References

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A combination of microgenomics approaches to understand the impact of gut microbiota on the regulation of the hypothalamo-pituitary-adrenal axis in rats

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Introduction

The gastro-intestinal tract hosts a complex microbial community, the gut microbiota, which is nowadays regarded as a full organ taking part in the host physiology. Recently, we showed in rats that the gut microbiota regulates the hypothalamo-pituitary-adrenal (HPA) axis reactivity, as reflected by a higher corticosterone systemic concentration in germfree animals than in conventional ones following an acute stress (Crumeyrolle-Arias *et al*, 2014). Our aim was to identify which HPA axis genes are regulated by the gut microbiota.

Material and methods

We subjected germfree and conventional rats to an acute stress, killed them by decapitation and collected and froze the brain, as well as pituitary and adrenal glands. Non stressed rats served as controls. Brain and adrenal gland sections were stained to localize areas of interest, and specific cell harvesting techniques were applied, according to the distribution and density of the key cells: micro-punching of the paraventricular nucleus (PVN) in the hypothalamus, laser capture microdissection (LCM) of cell clusters in the zona fasciculata of the adrenal gland cortex. The expression level of a panel of 48 genes, selected according to their role in the HPA axis activity and reactivity, was analyzed using TaqMan® Array Micro Fluidic Cards; qRT-PCR was carried out in both tissues and a DNA pre-amplification was applied in the adrenal gland cell clusters. The development of a distinct combination of techniques is in progress for analysis of the pituitary gland: immuno-labelling to identify the corticotropic cells which are scattered in the anterior lobe of the gland, LCM of 20 to 30 single cells followed by gene expression analysis involving DNA pre-amplification with the TaqMan® PreAmp Cells-to-C_T™ kit.

Results and discussion

First results indicate that gut microbiota regulates the expression of several HPA axis genes in the PVN and in the zona fasciculata of the adrenal gland cortex in both non stressed and stressed rats. In conclusion, this microgenomics workflow allowed us to address the issue of gene expression analysis in a heterogeneous set of tissues involved in a specific neuroendocrine pathway.

Reference

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This work was supported by Thermo Fisher Scientific-Life Technologies who kindly provided PicoPure RNA extraction kits, TaqMan® PreAmp Master Mix and Cells-to-C_T™ kits and TaqMan® Array Micro Fluidic Cards. We acknowledge Excilone for supporting LCM experiments.

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Comparative analysis of RNA sequencing methods for degraded or low-input samples

SATIJA R[1]

RNA-seq is an effective method for studying the transcriptome, but it can be difficult to apply to scarce or degraded RNA from fixed clinical samples, rare cell populations or cadavers. I will present our recent comparative analysis to determine optimal protocols for low-input and low-quality RNA-seq. We created and sequenced 10 libraries using different protocols on the same human RNA sample, and developed a set of computational metrics to evaluate and benchmark different techniques. We found that the RNase H method performed best for chemically fragmented, low-quality RNA, and can even effectively replace oligo(dT)-based methods for standard RNA-seq. Moreover, the SMARTer method successfully created libraries for ultra-low inputs, enabling the development of a protocol for single cell RNA-seq. I will conclude by demonstrating how we have applied these single cell methods to characterize enormous cellular heterogeneity, reconstruct regulatory circuitry, and discover novel cellular populations in the mammalian immune system.

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Low RNAseq microdissected plant tissue

BALZERGUE S[1], BORREGA N , YANSOUNI J, BRUNAUD V, DELANNOY E , FAURE JD , DUBREUCQ B[2]

Introduction

Plants are complex organisms whose growth involves coordinated development in space and time. In order to fully understand the molecular mechanisms occurring during development, this complexity needs to taken into account Laser assisted microdissection (LAM) is a method of choice to provide access to specific cell types. In this work we have coupled LAM with RNAseq to address the questions of RNA quality for quantitative transcriptomic studies.

Materials and Methods

Embedded *Arabidopsis thaliana* developing embryos have been microdissected using a ZEISS PALM microscope. Total RNA was extracted and quality checked. Libraries have been obtained using the SMARTer® Ultra™ Low RNA kit and RNAseq was performed using the Illumina Hiseq2000 machine. The data obtained were processed packages astqc for quality, bowtie2, samtools for reads mapping and count, and RSeQC for distribution along gene model.

Results and Discussion

We used 5, 0.5 and 0.1 ng of total microdissected RNA as starting materiel. The initial quality was critical since RIN indexes below 6 did not allow successful production of libraries. The number of mapped reads was very good and almost the same in all samples. More than 16000 genes were detected using 0.1 and 5 ng of RNA template and 11200 with 0.5ng. The data show an increase of poly A/T stretches in the reads when decreasing the RNA template quantity, together with a bias in the 5'-3' covering of the reads over gene bodies. On the same line the RNA-seq read counts reproducibility was lower for low input quantities.

Conclusions

We show that it is possible to perform RNAseq on as low as 0.1 ng microdissected plant total RNA templates. We can successfully identify plant gene expression in very small cell samples using this technique, but quantification of differences does not appear to be as straightforward and will need additional studies to estimate the technical variability.

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Genomic Characterization of Challenging or Limited Research Samples using the Ion AmpliSeq™ Targeted Re-Sequencing Technology

JACKSON S[1]

Introduction

Whole genome and exome sequencing has provided researchers a grand view of the genetic variations that occur in human tissues and cells. However, as clinical research techniques become more sophisticated, the need to analyze nucleic acids from very small amounts and very rare samples increases. Genomic analysis of genetic variance within these limiting sample sets may provide important insights to tumor biology, biomarker discovery, disease pathogenesis, and drug development in the future.

Ion AmpliSeq™ is a fast, simple, flexible, and extremely multiplex-able PCR-based target enrichment procedure for next generation sequencing. By performing a PCR preamplification before sequencing, hundreds to thousands of specific regions of the genome can be quickly analyzed from ≤ 10ng sample input. Furthermore, the PCR enrichment ensures Ion AmpliSeq™ is compatible with small, fragmented DNA, such as that recovered from archived FFPE samples or from plasma (where mean size of fragments is ~150 bp). Here we demonstrate the robustness and compatibility of Ion AmpliSeq™ with such DNA and we also demonstrate the utility of Ampliseq™ for analysis of DNA from low amounts of starting material (as obtained from FACS sorting, LCM, or HD-CTC captured cells) for research. Finally, we describe a sample-to-answer workflow that is compatible with small amounts of recovered nucleic acid from clinical research samples.

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Session 4: Microgenomics and Proteins

Chairman: Lance Liotta

Molecular profiling using Laser Microdissection, Protein Microarrays, and Genomics, for the Individualized Therapy of Metastatic Breast Cancer

LIOTTA LA[1], ESPINA V, PETRICOIN E, WULFKUHLE J, PIEROBON M

Metastasis is the major cause of breast cancer treatment failure. Genomic sequencing and proteomic pathway mapping of metastastic lesions has recently shown that the molecular factors driving the growth or drug resistance of metastatic colonies may be quite different from the primary tumor from which they are derived. Consequently, molecular profiling of the primary tumor may NOT accurately predict the optimal therapy that would be effective for potentially lethal metastasis. Microgenomic and microproteomic technology, including Laser Capture Microdissection (LCM), Reverse Phase Protein Micrarrays (RPPA), and Genomic Sequencing, have made it possible to conduct a new category of clinical trial to individualize the therapy of metastatic cancer. For these trials, the molecular profiling of metastatic breast lesions, not the primary tumor, is used as a starting point for individualized therapy of stage IV disease. The goal is to elucidate the deranged, mutated, or hyperactive molecular (proteomic and genomic) signaling network within metastastic lesions destined to progress in the face of currently available treatment. The hope is that gathering this information can a) provide individualized therapies that would not have been originally selected by the treating physician, and b) identify entirely new targets for therapeutic intervention. A first critically important technology used for these trials is LCM. Cellular heterogeneity of tumors hinders accurate molecular profiling for individualized therapy. For this reason, homogenization of a tumor surgical specimen or a biopsy will yield false negative and false positive data depending on the relative contribution of host cells and tumor sub-clones that are in unknown proportion in the tissue sample. LCM permits the procurement of tumor cells under direct microscopic visualization for proteomic and genomic analysis of tumor versus host (stroma) cells or phenotypically defined subpopulations of tumor cells. A second critical technology is RPPA. The phosphorylation, or activation state of kinase-driven signal networks contains important information concerning both the disease pathogenesis as well as potential for therapeutic target selection. Modulation of ongoing cellular kinase activity represents one of the most rapidly growing arenas in new drug discovery. Through the use of phospho-specific antibodies on the RPPA platform, it is now possible to quantitatively evaluate the state of entire portions of a signaling pathway or cascade, even though the cell is lysed, by looking at hundreds of signal proteins and kinase substrates at once. By measuring the proportion of those protein molecules that are phosphorylated, or activated, we can infer the level of activity of that signal node and the upstream and downstream members of the signal pathway. The activated signal pathway contains the drug target of interest. It is theoretically feasible to administer combination therapy targeting multiple interdependent points along a pathogenic pathway or targeting multiple distinct yet cooperating dysregulated pathways. The ultimate goal is tailored combination therapy of metastasis that provides higher individual efficacy with lower toxicity. Under the novel design of this trial (Side-Out Trial), a breast cancer patient's metastatic lesion is biopsied, microdissected with LCM, and profiled with proteomic and genomic technology. A treatment selection committee reviews the patient's data and recommends therapeutic options based on the molecular findings. Side Out Trial One is now completed. It successfully met its statistical goal of extending the progression free survival period to a ratio better than 1.3 compared to the progression free survival period for the individual patient's last prior therapy. The therapies selected based on molecular profiling for all 25 patients were different than the physician's choice without the profiling. This study demonstrates the feasibility of personalized cancer treatment for metastatic breast cancer using a first-of-its-kind highly multiplexed molecular profile based rationalized treatment recommendation. Based on the success of the first pilot Side Out Trial, Side out Trial number Two is now open as a multi-institutional consortium accruing patients with stage IV breast cancer.

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Oocytomics: combining transcriptomics and proteomics to understand post-transcriptional regulation in bovine oocyte

DALBIES-TRAN R[1], HENNEQUET-ANTIER C, CABAU C, ANGULO L, AUCLAIR S, PONSART C, MARSAUD N, LABAS V, UZBEKOVA S

Introduction

The last few days of oocyte development preceding ovulation are critical for its ability to sustain subsequent early embryo development. During these stages, the fully grown oocyte is essentially transcriptionally quiescent, and gene expression is mostly under post-transcriptional control, through cytoplasmic polyadenylation, deadenylation and possibly noncoding RNA targeted degradation. Combining transcriptomic and proteomic approaches, we are investigating the regulation of gene expression in the bovine oocyte and its contribution to oocyte developmental potential.

Material and Methods

Oocytes were collected post-mortem on slaughterhouse ovaries, followed when adequate by in vitro maturation, or in vivo by ovum pick-up after superovulation. For transcriptomics, RNA was extracted from pools of 15-20 oocytes and submitted to two rounds of amplification (Picopure RNA isolation kit and dual RiboAmp Plus kit (Invitrogen)). It was analyzed 1) using a non-commercial microarray (GEO GPL6695); 2) by RNAseq with a HiSeq2000 (Illumina). For qPCR, reverse-transcribed RNA equivalent to 0.05 oocyte was used. For proteomics, Intact Cells Matrix-Assisted Laser Desorption/Ionisation time-of-flight Mass Spectrometry (ICM-MS) was applied for single oocyte analysis without protein extraction, and nanoLC-MS-MS was used for protein identification.

Results and Discussion

About 300 proteins could be identified. The profile of these proteins and the corresponding maternal transcripts during oocyte maturation were compared. In addition to this global analysis, we considered well-known maternal-effect genes such as *NLRP5*, *OOEP* and *PADI6*, as well as additional genes suspected to play a role in embryo development. The RNA and protein profiles were analyzed in relation with the presence of putative post-transcriptional regulatory elements within the 3' untranslated region.

Conclusions

Combining transcriptomics and proteomics is a promising methodology to delineate the complexity of post-transcriptional control of gene expression in the fully grown mammalian oocyte. Current approaches will be complemented by the transcriptome of small nonconding RNA in the oocyte.

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Combining surface plasmon resonance and mass spectrometry to identify Bone Morphogenetic Protein (BMP) interactant

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Introduction

At the pituitary level, BMP, members of the transforming growth factor ß (TGFß) superfamily, govern pituitary organogenesis but also play roles in different differentiated cell types. For instance, in gonadotrope cells involved in reproduction function, the regulation of follicle-stimulating hormone (FSH) synthesis is affected by BMP. Several BMP ligand mRNAs are present in sheep pituitary suggesting that BMP can exert paracrine/autocrine actions on FSH synthesis. However, the variations in FSH β mRNA and plasma FSH concentrations across the estrous cycle or *in vitro* are not associated with changes in the expression of BMP4 and BMP receptor mRNA (Sallon *et* al. 2010). The aim of the study was to determine whether ovine pituitary cells produced BMP and/or BMP inhibitors and then to identify them.

Material and Methods

The potential presence of BMP or BMP inhibitors in supernatants from ovine pituitary cells cultured for 48h was investigated by using a bioactivity test based on embryonic mesenchymal cells (C3H10T1/2 cells) transfected with a BMP-responsive element fused to firefly luciferase reporter gene. To determine whether the pituitary cell supernatants exhibiting an inhibitory activity for BMP action were able to bind BMP, BMP-4 (between 50 and 100 ng) was immobilized on a BIACORE sensorchip and supernatants (from around 5000 cells) were injected on the chip for analysis by surface plasmon resonance. The bound factor was then recovered and analysed by nanoLC-MS/MS.

Results and Discussion

The BMP bioactivity assay shows that pituitary supernatants contain factor(s) able to inhibit BMP action. Molecular interaction analyses using Biacore demonstrated that factor(s) contained in pituitary supernatants bound BMP-4. Recovery of the interactant fraction (around 40 ng) led to identify one molecule, thrombospondin-1, using mass spectrometry. Subsequent analyses confirm that thrombospondin-1 is produced by pituitary cells and is capable to block BMP-4 action.

Conclusions

By combining surface plasmon resonance and high-resolution mass spectrometry, we identify a factor produced by pituitary cells which antagonized BMP action. This strategy can be broadly applied to establish the pattern of factors, present in biological samples, interacting with a known molecule.

References

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Mass Spectrometry Imaging coupled to Microproteomics: From Imaging to identification of proteins on tissue section

WISZTORSKI M[1], FRANCK J[1], QUANICO J[1], FATOU B[1], DEMONS A[1], SALZET M[1], FOURNIER I[1]

Introduction

Novel and sensitive strategies such as MALDI MS imaging (MSI) allow the precise localization of exogenous and endogenous compounds directly on tissue section and allow the study of interaction between cells and the direct environment [Franck 2009]. Nevertheless, for proteins, direct identification of biomarkers on-tissue by *in situ* strategies remains difficult and only allows access to a limited number of major proteins.

Material and Methods

This presentation will briefly depict MALDI MSI through different applications such as exogenous compounds, metabolites, peptides/proteins localization but also nucleic acids like mRNA. Aspect of sample preparation analysis will be exposed and strategies for in situ identification will be discuss. In particular, in order to improve proteins identification from tissue sections, we recently proposed a hybrid strategy using Liquid Micro Junction to extract tryptic peptides coming from localized on-tissue digestion followed by a LC MS & MS/MS analysis leading to the identification of thousands of proteins [Quanico 2013, Wisztorski 2013].

Results and Discussion

Localization of compounds coming from various source of tissue such as plants, invertebrates or mammals will exemplify the large panel of applications for which MALDI MSI can be used. We will also illustrate how recent improvements for proteins identification will open the gate of this technique for clinical applications in particular for cancer research.

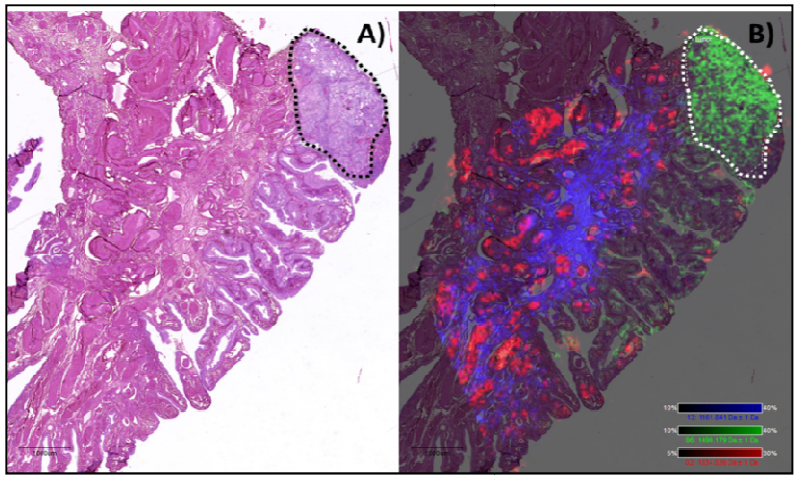

Figure 3 : A) Picture of Fimbria tissue section with HPS staining. B) Molecular information obtain using MALDI MSI showing three compounds with different localizations. In green, a compound marker of fimbria tumor.

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Unlocking the prognostic significance of microdissected proximal lesions from the human colon

DREW DA, DEVERS T AND ROSENBERG DW[1]

Despite increased implementation of screening colonoscopy, interval cancers in the proximal (right) colon remain a major public health concern. This observation underscores the limitations of current screening strategies and the critical need to develop advanced endoscopic techniques. The presence of aberrant crypt foci (ACF), the earliest morphologically identifiable epithelial abnormality in human colon, may serve as a surrogate marker for cancer risk, but the significance of this lesion within the proximal colon has not been addressed. Our laboratory has implemented high-definition chromoendoscopy on ~ 200 normal subjects to characterize the neoplastic potential of proximal ACF. The development of a combinatorial approach to maximize genomic and molecular data acquisition from ACF has been necessitated due to their exceedingly small size (< 5mm). In the following study, methods are described that combine UV/IR and laser-capture microdissection with an ultrasensitive nanofluidic proteomic immunoassay and DNA mass spectrometry. These highly sensitive methodologies enable the interrogation of the mutational spectrum of microscopic biospsies removed from the human colon and to interpret post-translational modifications of MAPK within the context of underlying oncogenic changes. Among the somatic mutations that have been identified include mutations to APC^{R876*} and $FLT3^{I836M}$, as well as an insertion/deletion with the EGFR gene, mutations that are related to the underlying pathology of the lesion. Finally, microdissection has been combined with a powerful genome-wide methylation analysis to begin to uncover the methylation state of early neoplasia within the proximal colon.

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POSTERS

Photosynthesis mediates expression of *Sorghum bicolor* transporters in arbuscular mycorrhizal symbiosis

ARNOULD C[1], BRULÉ D, GEAY L, KOEGEL S, COURTY PE, WIPF D

Introduction

Arbuscular mycorrhizal (AM) symbiosis is formed between soil fungi (*Glomeromycota*) and the majority of land plants. Plants received phosphorus (P) and nitrogen (N) via two different pathways. The direct pathway relies on P and N transporters located in the root epidermis. The mycorrhizal pathway involves the transfer of nutrients via AM-inducible P and N transporters located at the plant-AM fungal interface, namely arbuscules. In return, as AMF are obligate biotrophs, host-derived carbon (C) is transferred as monosaccharides from the plant to the AMF via the same interface. In our model plant *Sorghum bicolor* (Sorghum), we have characterized N, P and monosaccharide transporters involved in the direct and mycorrhizal pathways. Here, we modified the photosynthetically fixed C of Sorghum by applying three different light treatments: full light, shadow and dark.

Material and Methods

An Arcturus XT microdissection system was used to collect a total of 5000–15 000 cells for each sample. RNA from the collected cells was extracted using Pico Pure RNA isolation Kit and quality of the extracted RNAs were verified using a bioanalyzer. Synthesis of cDNA and quantitative reverse transcriptase polymerase chain reaction analysis was done using the iScript cDNA Synthesis kit. Gene expression was quantified by qPCR analysis.

Results and Discussion

The AM-inducible P and monosaccharide transporters are over-expressed under shadow and dark conditions whereas the AM-inducible N transporter is over-expressed under full light and shadow conditions. Of interest, one non AM-inducible monosaccharide transporter is over-expressed under dark condition only.

Conclusions

In AM and non-AM Sorghum plants, the expression of N, P and monosaccharide transporters is mediated and is adjusted regarding C allocation.

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Proteomic analysis of sub-retinal deposits in age-related macular degeneration

ARNOULD C[1], LUCCHI G, SIMON E, LECLERE L, BUTEAU B, PECQUEUR D, TRUNTZER C, DUCOROY P, LHERMINIER J, BRETILLON L

Introduction

Age-related Macular Degeneration (AMD), one of the major causes of visual loss in Western populations, originates from defects of the retinal pigment epithelium (RPE). AMD affects the macula, the central zone of the retina that is involved in the vision of details and colors. The deposition of cellular debris beneath the retina is well recognized as a key element in the events leading to AMD, by creating a physical barrier between the retina and the vascular choroid. However, the composition of those deposits remains largely unknown. In order to improve knowledge on the pathophysiology of retina aging, we characterized age-related proteome changes in the retina and RPE of the ApoB100$^{+/+}$,LDLR$^{-/-}$ mouse, as a model that develops the main clinical features of human retinal aging.

Material and Methods

Ocular globes of aged and control mice were embedded in OCT, and cryosectioned. Laser Capture Microdissection was used to collect outer segments of the photoreceptors, retinal deposits and RPE samples. After protein extraction and in-gel trypsin digestion, recovered peptides were concentrated and analyzed by nanoLC-MS/MS Data were processed using a set of home-made and open-source software tools. Differentially-accumulated proteins were selected using the MSstats library on R-software with a 5% FDR.

Results and Discussion

From 38 to 96 proteins were identified in the different samples. Lower levels of retinol dehydrogenase and up-accumulation of S-arrestin were found in ApoB100$^{+/+}$,LDLR$^{-/-}$ mice compared to controls. The concomitant up-accumulation of crystallins is consistent with proteomic data in eyes collected from patients with AMD showing increased levels of crystallins.

Conclusions

The proteomic data add possible clues to explain functional changes in the ApoB100+/+, LDLR-/- mouse.

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Laser Capture Microdissection combined with Maldi-T of mass spectrometry to demonstrate that s1-casein is required for efficient export of the other caseins from the endoplasmic reticulum to the Golgi apparatus

BEAUVALLET C[1], BEVILACQUA C[1], CHANAT E[2] AND MARTIN P[1]

Introduction

Caseins, the main milk proteins, aggregate in the secretory pathway of mammary epithelial cells (MEC) into large supramolecular structures, the so-called casein micelles. The role of individual casein in this process and the detail structure of the casein micelle remain poorly understood. In the goat species, a deficit in s1-casein is responsible for the accumulation of caseins in distended rough endoplasmic reticulum (RER) cisternae and for a perturbation of the secretion process of proteins and lipids that are synthesized as triglycerides at the ER level. In contrast, no accumulation was recorded in MEC lacking -casein expression.

Materials and Methods

To unravel the underlying mechanisms of the casein secretory pathway and to go further into the understanding of the primary steps of casein micelle formation, acini from goats of extreme genotypes at the *CSN1S1* locus (A/A vs. O/O) that codes for α_{s1}-casein, were captured using Laser Microdissection (Veritas, Arcturus, Life Technologies) and directly analyzed using MALDI-Tof Mass spectrometry (Voyager DE STR, Applied Biosystems). A differential proteomic analysis (2D-DIGE) of milks from such goats was also carried out to assess the impact of the disturbed secretory process on milk protein composition. Micellar caseins were removed by centrifugation and the supernatants labelled with the three CyDyes, of which one (Cy2) was used as internal standard (equimolar mix of each sample).

Results

MALDI-Tof MS analysis of microdissected MEC revealed specific peaks, in the casein-size region, only in spectra corresponding to s1-casein deficient cells (goats CSN1S1 O/O). We show that members of the casein family, namely s2-casein, β-casein and κ-casein, accumulated significantly in MEC from these goats. Interestingly, these caseins were in their immature forms (unphosphorylated) confirming their ER origin. In addition, a hundred of spots were significantly discriminated between milk from genotypes CSN1S1 A/A and O/O by 2D-DIGE. The most differential spots observed in O/O milks were identified from peptide mass fingerprinting (MALDI-Tof MS) as ER-resident proteins, strongly suggesting a singular secretory mechanism for this genotype.

Conclusions

Our data suggest that s1-casein interacts with the other caseins in the rough endoplasmic reticulum and that the formation of this complex is required for their efficient export to the Golgi apparatus.

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GABI core facility:An integrated approach from tissues to biomolecules

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Genomics has opened a new era to understand genes' functions and interactions, and identify gene networks regulating animal performance. Research in integrative biology within GABI is combining genomics (sequencing, transcriptomics, qPCR) with tissue and cell biology, using original phenotypes or well-known models such as the epithelial cell of the mammary gland or the skin melanocyte in a spontaneous cutaneous melanoma in pigs. Functional genomics has to deal with the diversity of cell types in a complex tissue. Whole tissue approach provides a global view of the function, but understanding fine regulations requires to separate cell types. A major challenge for cell-specific genomics (also called microgenomics) is to identify and separate cell types in a way preserving the integrity of the biomolecules (DNA, RNA, proteins) which need to be further characterized, taking into account the limited quantities obtained. Two laser capture microdissection systems VERITAS and XT (Arcturus, Life Technologies) combine LCM and UV laser cutting for ultimate microdissection flexibility and a new equipment will become available before the end of 2014 for high-throughput qPCR. Processes for handling biological samples, for the quality control of biomolecules, for laser microdissection and microarray functional analysis are certified ISO:9001 and are part of the @BRIDGe facility recognized as national strategic by INRA for animal genomics. @BRIDGe is part of CRB-Anim, a national infrastructure supported by ANR for the 'Investissements d'avenir' programme.

GABI facilities have developed protocoles for cell types of interest to GABI and are making this expertise available to external users who come to us with new questions regarding a specific tissue. Protocoles have been validated for RNA isolation from milk fat globules to profile mammary epithelial cell expression during lactation and transcriptional response to a bacterial infection, and from melanocytes/melanoma cells in order to conduct a transcriptomic study of predisposition to melanoma in pigs from a small number of cells (a hundred). The facility benefits from the expertise and the network established in medical sciences, by our partner Excilone, with public institutions and private companies (Pierre Fabre, L'Oréal). As an exemple, a partnership between our microgenomic facility and G. Lezmi (INSERM, Hopital Necker, Paris) led to implement LCM on epithelial and mesenchymal lung tissues for MAKP disease (a congenital lung pathology) to compare gene expression between healthy and pathological tissues. Each project dealing with a new tissue or cell type needs a preliminary feasibility study, and the range of tissues studied at GABI facilities is likely to increase thanks to external collaborations. Recently we implement a feasability test to characterize at the molecular level tumor effusions with P. Vielh (Inserm U981, Institut Gustave Roussy, Villejuif).

For more information, please contact us at gabi-abridge@listes.inra.fr.

Some recent results :

Bevilacqua C, Makhzami S, Helbling J-C, Defrenaix P, Martin P, 2010. Maintaining RNA integrity in a homogeneous population of mammary epithelial cells isolated by laser capture microdissection. *BMC Cell Biology* 11:95.

Brenaut P, Bangera R, Bevilacqua C, Rebours E, Cebo C, Martin P, 2012. Validation of RNA isolated from milk fat globules to profile mammary epithelial cell expression during lactation and transcriptional response to a bacterial infection. *J Dairy Sci.* 95:6130-6144.

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Transcriptome Analysis using Ovation® Single Cell RNA-Seq System

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Introduction

Expression analysis of mixed populations of cells masks the heterogeneity that exists within that population. Enabling a better understanding of these dynamics at the level of individual cell is vital to understanding the development of tissues and disease progression. An ideal analytical tool to study single cells within populations requires high specificity, sensitivity and low input requirements.

Material and Methods

In this study, we use the NuGEN Ovation® Single Cell RNA-Seq system to perform single cell transcriptome analysis of input amounts as low as 10 pg purified total RNA. Briefly, first strand cDNA synthesis was carried out from serial ten-fold dilutions of Universal Human Reference (UHR) and First Choice® Human Brain Reference RNA using proprietary NuGEN whole transcriptome primers included in the Ovation® Single Cell RNA-Seq system. Those primers enriched for non-rRNA sequences in the transcriptome, producing libraries with low rRNA content. Barcoded libraries were mixed in equimolar ratios prior to sequencing on an Illumina sequencer, and the resulting sequencing reads were aligned against the hg18 reference genome using Bowtie read aligner.

Results and Discussion

Data analysis showed high percentage of aligned reads for all inputs, even 5' – 3' transcript coverage and more than 34% of exon RefSeq counts. In addition, directional cloning of the cDNA during second strand synthesis resulted in NGS libraries with almost 100% strand retention, illustrating the utility of the System to detect antisense expressed transcripts. Comparison of the number RefSeq genes detected at different FPKM thresholds between samples showed that a large proportion of RefSeq genes are identified with only 10 pg of total RNA, which represents the average RNA amount of a single vertebrate cell. We also evaluated the effect of different lysis methods and the impact of residual gDNA in the library construction method.

Conclusions

The effectiveness of libraries prepared with the Ovation Single Cell RNA-Seq System for analysis of differential gene expression will be discussed.

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Transcriptome analysis of CA1 hippocampal area in a mouse model of Alzheimer Disease

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Introduction

Alzheimer disease (AD) is the most widespread dementia. Amyloid-β (Aβ) peptides accumulating in the brain of patients are assumed to be neurotoxic and key actors in the neurodegenerative process of the disease (Duyckaerts *et al*., 2009). The APPxPS1-Ki mouse is a unique transgenic model as it develops classical extracellular lesions but also intraneuronal Aβ–accumulation combined with selective cell loss and cognitive impairments in hippocampal-dependent tasks (Faure *et al*., 2011). In order to decipher the impact of Aβ-accumulation on gene expression, we performed a transcriptome analysis of CA1 hippocampal area from APPxPS1-Ki versus PS1-Ki mice.

Material and Methods

CA1 regions were laser-microdissected (Leica-LMD7000) from five snap-frozen brains of APPxPS1-Ki and five PS1-Ki mice. RNAs were extracted (Macherey-Nagel), labeled and hybridized on whole genome microarrays (Agilent Technologies). Raw data were analyzed with Mapix software (Innopsys) and quantile-normalized (R freeware). T-test was performed to identify differentially expressed genes and Gene Ontology Enrichment was calculated using Gorilla (http://cbl-gorilla.cs.technion.ac.il/).

Results and Discussion

Microarray data analysis allowed for identification of 254 genes up-regulated and 138 down-regulated in CA1 region between APPxPS1-Ki and PS1-Ki mice. We calculated Gene Ontology Enrichment to determine over-represented GO categories. Interestingly, we reported an enrichment of categories corresponding to synapse organization, apoptotic pathway and neuron activity. This brought some important clues to explain cell loss and synaptic dysfunctions observed in mice as well as target genes for lentiviral approaches.

Conclusion

Transcriptome analysis of CA1 area allowed for identification of specific genes and pathways deregulated by Aβ-accumulation. This might help to better understand mechanisms of AD progression and identify potential candidates to refine therapeutical assays.

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Cell Heterogeneity among *Bacillus cereus* Population during Cold Adaptation

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Introduction

The adaptive ability of microorganisms is generally studied at the population scale, and expressed as the mean behavior of a whole clonal population. In these conditions, the *cshA* gene, encoding for a RNA helicase, was shown to be overexpressed and required during cold growth in *Bacillus cereus* (1). This spore-forming soil bacterium can grow in refrigerated food and consequently cause food-poisoning.

More recently, news technics allowed identifying single-cell heterogeneity that may generate sub-populations with phenotypic differences among a clonal bacterial population.

The aim of this study was to determine the expression level of *cshA* at the cell scale during *B. cereus* growth at low temperature.

Material and Methods

A transcriptional fusion between the *cshA* promoter and a *gfp*-reporter gene was constructed on a low-copy number plasmid and introduced into *B. cereus* before growth at optimal or at low temperature. A similar transcriptional fusion with a constitutively expressed promoter was used as a control. Epifluorescence microscopic observations of single-cells were performed over time during the kinetics of the bacterial growth (lag phase, exponential phase and stationary phase).

Results and Discussion

The *cshA* promoter was activated whatever phase of growth or incubation temperature, but the observed mean fluorescence was higher during the lag phase of cells grown at low temperature. This result suggests that *cshA* is particularly important during cold adaptation of *B. cereus* cells.

Heterogeneity of the fluorescence level was observed among cells, for both control and *cshA* promoter, in all tested conditions, suggesting phenotypic noise in GFP expression. However, a higher heterogeneity was observed for the *cshA* promoter during the lag phase at low temperature. This may indicate that only a small fraction of the population overexpress *cshA* and can therefore adapt efficiently to low-temperature.

Conclusions

Further studies are needed to understand the role of the heterogenic expression of *cshA* among *B. cereus* population at low temperature.

References

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Impact of low read count genes filtering and gene-based analysis of single-end RNAseq data in a physiological context

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Introduction

RNA-sequencing (RNAseq) is often applied to populations of apparently hormogenous cells cultured in standardised conditions therefore limiting intra-group variability. Applying RNAseq analysis to cells derived from *in vivo* settings in contrasting physiological conditions implies an increased complexity of signals and greater heterogeneity of cell populations making data analysis more challenging. Thus, the analysis methodology is critical for optimal retrieval of significant results. Small changes in gene expression, typical of physiological responses, can render these problems more critical but should not be ignored (1). We present here a RNAseq analysis methodology implemented for addressing the influence of hypothyroidism on neurogenesis in the adult mouse.

Material and Methods

Specific brain areas were laser microdissected using a Leica LMD6500. Single-end libraries were constructed and sequenced with Illumina Truseq and Illumina NGS HiSeq system. Mapping was performed with Bowtie 0.12.7. DESeq 1.10.1 was used for differential analysis.

Results and Discussion

After the removal of all duplicated reads and multiple hits to increase the stringency, we analysed the impact of filtering low read count genes (LRC, <5 reads per gene) before performing differential analysis. LRC filtering removes about half of annotated genes. More importantly, this step allows identification of more differentially expressed (DE) genes between conditions (hypothyroid verus euthyroid) and these comparisons can show lower fold-changes than those gene sets that lose significance, proving the greater sensitivity of the differential analysis (Figure 1).

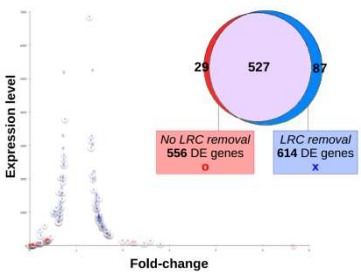

Figure 1. Impact of low read count genes renewal on differentially expressed (DE) gene numbers.

Conclusions

After performing adequate filters, LRC removal leads to more significant results with physiological data. Differential analysis at the gene level, as opposed to a transcript based analysis, is better to deal with single-end RNAseq results as a first approach. Validation by qPCR is ongoing to consolidate these findings.

References

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Deciphering β-arrestin-dependent signaling by RPPA

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Introduction

Phosphorylation events play a major role in G protein-coupled receptors (GPCRs) signaling upon ligand binding. Besides the classical G protein-dependent pathway, -arrestins scaffolding proteins have been shown to activate key signaling actors such as Src or MAP kinases. We previously described a highly sensitive method to study protein phosphorylation pattern by RPPA (Dupuy et al). Here, we took advantage of it to compare 3 GPCRs (i.e.: FSH-R, 5HT2C-R and 5HT4a-R) previously shown to signal through β-arrestins via different mechanisms.

Material and Methods

GPCRs were transiently expressed in HEK293 cells and endogenous β-arrestins (isoform 1, isoform 2 or both) were silenced using siRNA. Three days after transfection, cells were stimulated with their cognate agonist (FSH or 5HT). For each receptor, 5 independent dose-response (6 points) and kinetics (0 to 120 min) were carried out for four siRNA conditions. Samples were spotted on nitrocellulose-coated slides and RPPA were performed with multiple antibodies as previously described (Dupuy *et al*).

Results and Discussion

Seven hundred twenty experimental conditions were compared per slide. RPPA measurements correlated with Western blot analyses made on samples from the same experiments (r^2=0.72, 0.85 and 0.72 for FSH-R, 5HT2C-R and 5HT4a-R, respectively). Our data indicate that β-arrestins are involved in ERK, CREB and rpS6 phosphorylation by the 3 GPCRs, but the magnitude and the β-arrestin isoform specificity are receptor specific. More antibodies will be tested using RPPA and, since they come from a unique sample set, data could be cross-validated and compared.

Conclusions

Most classical phosphoproteomic approaches are limited to capture the subtle and highly dynamic nature of phosphorylation cascades. RPPA analysis allowed us to generate a large-dataset with high throughput that is well suited to build ODE-based dynamical models of intracellular signaling networks.

References

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Expression bimodality in the insect pathogene *Xenorhabdus* using single cell technologies

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Introduction

Xenorhabdus nematophila is a motile Gram-negative bacterium wich forms a mutualistic association with a nematode and is highly pathogenic for a wide range of insect larvae. Flagellum biogenesis and bacterial motility require the co-ordinated transcription of more than 50 genes organized in three hierarchically classes. The flagellar network from *X. nematophila* also controls expression of several virulence genes such as hemolysins, lipases and proteases[1].

Material and Methods

In this study, a *fliZ* mutant (a *Xenorhabdus* regulator protein of flagellar regulon) was constructed. Wild-type strain and *fliZ* mutant transcriptome were analysed by RNA-Seq. Bimodal expression was observed using single-cell analysis. Finally, fluorescence-activated cell sorting (FACS) and RT-qPCR quantification were conducted.

Results and Discussion

RNA Seq analysis identified FliZ as a global regulatory protein controlling positively or negatively the expression of 278 genes. FliZ is required for the efficient expression of all flagellar genes, probably through its positive feedback loop[1]. FliZ also up- or downregulates the expression of numerous genes encoding non-flagellar proteins. Effectively, *Xenorhabdus fliZ* mutant displays no hemolytic and lipase activities. Single-cell analysis revealed the bimodal expression of several markers of the FliZ regulon. In addition, the combination of FACS and RT-qPCR quantification showed that this bimodality generated a mixed population of cells either expressing ("ON state") or not expressing ("OFF state") FliZ-dependent genes[2].

Conclusions

FliZ plays a key role in cell fate decisions, by creating individuals with different potentials for motility and host interactions.

References

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Comparison of mRNA expression in gill ionocytes from trout adapted to freshwater or seawater using laser microdissection capture

LEGUEN I[1], MONTFORT J, LE CAM A, PERON S, FAUTREL A

Introduction

Fish gill is a very complex organ implicated in several functions such as respiration, maintenance of hydromineral and acid-base balances. Gill ionocytes are important cells implicated in ion exchanges between internal and external medium (freshwater/seawater) to maintain blood ionic homeostasis. These differentiated cells absorb salts in freshwater but secrete the same salts in seawater. Ionocytes have an important plasticity during fish transfer to different salinity: they have to modify their ionic exchanges. These cells being scarce in gill organ (less than 10%) and hard to dissociate, their gene expression in freshwater, seawater and during salinity transfer is poorly understood. Our objective was to compare mRNA expression of freshwater and seawater gill ionocytes from microdissected cells.

Material and Methods

Gill from rainbow trout adapted to freshwater or seawater were frozen or RCL2-fixed and paraffin-embedded. Ionocytes, identified with a specific antibody, were collected on gill sections with a laser capture microdissection equipped with an infrared laser. After extraction, the quality of mRNA was checked, and then they were amplified before qPCR and microarray studies.

Results and Discussion

Several tests have been realized to find the best ways to obtain enough mRNA from gill ionocytes with good quality. Morphological analysis and immunolocalisation of ionocyte were similar for frozen or paraffin gill section, however mRNA quality was better in frozen section. A rapid immunostaining protocol was adapted to avoid mRNA degradation and loss. After the validation of mRNA amplification kit by qPCR on some ion transporters, hybridization on microarray has allowed to identify several genes differentially expressed between freshwater and seawater ionocytes. Some new ionic transporters have been characterized in freshwater and validated by qPCR or in situ hybridization.

Conclusions

The laser capture microdissection of trout gill ionocytes has been developed and has allowed to identify different mRNA expression between freshwater and seawater ionocytes. In perspective, it will be interesting to follow mRNA changes during transfer from freshwater to seawater.

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Microtranscriptomics of moth pheromone sensitive sensilla

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Introduction

Insect antennae are devoted to chemosensory functions (olfaction and taste). They carry morpho-functional units, the sensilla, each responding to a specific set of stimuli. In male moths, many are devoted to sex pheromone reception (Figure 1), that they use to locate females for mating. In order to understand the molecular mechanisms underlying pheromone reception, our objective is to identify genes specifically expressed or enriched in these structures, among which pheromone-binding proteins (PBP) and pheromone receptors.

Material and Methods

We used laser capture microdissection (LCM, Arcturus, ICE Platform, INRA Jouy-en-Josas) to collect pheromone-sensitive sensilla from *Spodoptera littoralis* male antennae (Lepidoptera, Noctuidae) for RNA extraction. In parallel, RNA was extracted from whole antennae. These RNAs were used as templates for qPCR and RNAseq (IBENS Platform, ENS Paris).

Results and Discussion

Candidate gene approach

We previously identified 3 candidate PBPs and a co-receptor proposed to be necessary for pheromone receptor functioning (Legeai et al 2011). qPCR analyses revealed that two PBPs were enriched in pheromone-sensitive sensilla whereas the co-receptor was down-regulated, suggesting it is not necessary for pheromone receptor functioning in S. littoralis.

Global approach

Illumina reads were assembled to construct a reference transcriptome. The reads were further mapped on the reference and gene expression profiling is in progress.

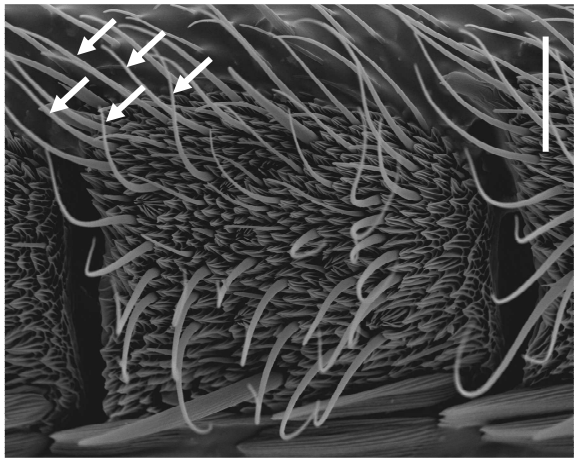

Figure 4 Scanning electron microscopic view of a segment of a *S. littoralis* male antennae. Some olfactory sensilla, or "micro-noses", are indicated by white arrows. Bar: 50µm.
Photo : M.C. François and C. Monsempes

Conclusions

We propose a mechanism as follows: 1) two PBPs bind the pheromone molecules, allowing them to cross the sensillum lymph and reach the receptors at the neuron membrane, 2) pheromone receptors function without the need of the co-receptor. We expect other factors to be revealed by the global transcriptomic analysis currently in progress.

Fundings: ANR-09-BLAN-0239 "LepidOLF"

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Transcriptomic analysis of neuroepithelial stem cells in the optic tectum of medaka

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Introduction

Contrary to mammals, **teleost** display a life-long and brain -wide neurogenesis making them an attractive model to study this process. In the optic tectum, neurogenesis occurs at the periphery in a niche called the **peripheral midbrain layer** (PML), which is composed of **neuroepithelial stem cells** (NeSCs). To gain further insights into biology of NeSCs of medaka, a transgenic line with GFP positive PML cells has been established, after *in vivo* enhancer screen, and used for gene profiling expression analyses.

Material and Methods

In vivo enhancer screen: consisted in testing putative regulatory elements located at the *wdr12/nop58* locus, two genes strongly expressed in NeSCs. RNAseq analysis: Cell dissociation was done after tectum dissection using FACSmax™ Cell dissociation solution. GFP+ and GFP- cells sorting from 1-89 and EMBL1 tecta was performed on a Facs MoFlo ASTRIOS (Beckman-Coulter).Total RNA was extracted using ARCTURUS® PicoPure® RNA Isolation Kit. We used TotalScript™ RNA-Seq Kit (Illumina) to generate RNA-Seq libraries. Libraries were sequenced for 50pb single read using a Hiseq1000 Illumina sequencer. Bioinformatic analysis: gene reads were mapped onto Oryzias latipes genome release 73. Global mapping statistics given by read alignment file were performed using a combination of htseqcount software and homemade algorithms.

Results and discussion

In vivo enhancer screen revealed an element encompassing the *wdr12* promoter driving the expression of the GFP in the NeSCs (1-89 line). Regarding to RNAseq, used protocols have been validated by our preliminary analyses. Following cell dissociation with **FACSmax ™** , **cells sorting** and RNA extraction we obtained from **few cells** (6000 to 20000) a **high quality RNA** (RIN>8). Bioinformatics analyses revealed the efficiency of the TotalScript™ RNA-Seq Kit that produces RNA-Seq libraries from **500 pg total RNA** using the " **tagmentation** " process. Count reads were found equally distributed on the whole genome for which we obtained a good reproducibility between biological replicates, moreover preliminary *in situ* hybridization analysis confirms RNA seq results.

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Custom High Fidelity, Targeted Transcript Depletion from Stranded RNA-Seq Libraries

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Introduction

For RNA expression analysis, NGS-mediated RNA-Seq offers greater sensitivity and higher resolution than microarrays. Unlike microarrays, all transcripts are capable of being detected, not just those for which probes have been designed. Unless they are depleted, however, highly-abundant RNAs such as ribosomal RNAs will account for a majority of reads, greatly increasing the cost of generating "informative" reads. Traditional methods of reducing these reads include: poly A+ selection or oligo dT priming, where informative non-polyadenylated transcripts are not represented, or "hybrization-based capture" which requires larger inputs, is time consuming, and may alter the relative transcript abundance by non-specifically binding and removing desired transcripts.

Material and Methods

NuGEN Technologies has developed a highly-effective method for selectively removing unwanted transcripts that is seamlessly integrated into the library generation process. This method, Insert-Dependent Adaptor Cleavage (InDA-C, pronounced *IN-da-see*) renders unwanted sequences incapable of being amplified. The InDA-C approach is independent of the nature of the first strand primers used or the source of RNA. Libraries can be prepared from total RNA primed with random primers, oligo-dT primers, or a combination of both. This enables all transcripts to be represented without the high background of non-informative sequences. Simple customization allows this method to be equally effective with essentially any RNA population. Depending on the tissue and species under investigation, there are many examples of "unwanted" abundant RNAs. Custom InDA-C probe sets can be designed at relatively low cost and with rapid turn-around time.

Results and Discussion

Data will be shown highlighting the effectiveness of InDA-C treatment in depleting "unwanted" transcripts for several custom designs (rRNA from plants, insects, model organisms; abundant transcripts in muscle, leaf, mammary tissue, etc).

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RNA quantification using high throughput real-time quantitative PCR

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Introduction

We recently acquired a Fluidigm Biomark HD system, available as a high throughput quantitative PCR platform. When compared to traditional real time PCR technologies, the Biomark HD System and the microfluidic chips from Fluidigm offer a considerable improvement in terms of capacity, allowing researchers to produce more data in less time (up to 9216 simultaneous qPCR reactions). High throughput qPCR platform raises several questions in terms of sensitivity (minimal input), absolute quantification and data processing.

Material and Methods

To address these questions, we designed experiments with scales of zebrafish total RNAs as samples, and Taqman probes as assays. RNAs were reverse transcribed with various RT enzymes to compare their sensitivity. In order to precisely quantify cDNA molecules, amplicons were cloned in plasmids and used as calibration curves to calculate PCR efficiency[1] for each probe. This process was automated thanks to dedicated R[2] scripts. We also investigated and objectively compared various methods[3,4] that aim to perform absolute quantification from individual amplification reaction.

Results and Discussion

1) Following RT enzyme and gene expression level, a minimum of 10 pg of total RNA seems to be a lower limit.
2) Quantification from individual profiles based on known data analysis methods are sensitive to both the baseline estimation and the search of the "window of linearity".
3) R software is greatly helpful to process large sets of data

Conclusions

We propose a comprehensive solution to perform high throughput qPCR and data analysis. Our framework integrates previously designed methods for quantifications and comparison as well as some improvements.

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Systems Biology Using Multi-Level Data from Single Cells

RUFF, D1

Introduction

Systems biology endeavors to understand the complex nature of life by using multi-dimensional approaches to interrogate biological processes. Employment of single cells offers great advantages for systems biology modeling. By analyzing the intricate properties of networks on an individual cell-by-cell basis, deciphering the interplay between genomic components and expression signatures can be greatly facilitated. Recent technological advances using microfluidics have enabled ready access to profile gDNA, mRNA, miRNA, and proteins as they orchestrate biological responses in single cells.

Material and Methods

For proof-of-principle experiments, the Fluidigm® C_1™ Single-Cell Auto Prep System captured individual cells from selected human cell lines into integrated fluidic circuit (IFC) chips. Single-cell lysates were processed in the C_1 IFCs for gDNA, mRNA, miRNA or protein interrogation, using whole genome amplification (WGA), template-switching mRNA seq, 377-plex miRNA reverse transcriptase cDNA conversion, or 92-plex proximity extension assay (PEA), respectively. WGA and whole transcriptome libraries were analyzed using next-generation sequencing readouts, and miRNA and protein PEA signals were detected by qPCR.

Results and Discussion

Isolated human single cells exhibited great diversity of mRNA expression levels as determined by mRNA seq data analysis. We routinely obtained expression data from 5,000-10,000 transcripts at a reading depth of 0.5-3 million reads per cell. Many mRNAs exhibited highly divergent expression from cell to cell. Somatic mutation identification by targeted resequencing for a panel of cancer genes showed low rates of *in vitro* evolution in short-term cultures. The detection of proteins generally correlated to detection of mRNA seq signal in each cell line, and the relative levels of mRNA to protein will be discussed.

Conclusions

This study demonstrates the ready application of the Fluidigm C_1 System to gather datasets for systems biology investigations. Our results confirm previous reports of the dramatic differences in RNA and protein expression levels at single-cell resolution in seemingly homogenous cell populations. By combining new workflows for gDNA, RNA, and protein profiling for the same individual cell, we foresee an emerging toolkit to enable the discovery of multi-omic interaction modules in systems biology.

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Absolute single-molecule sensitive next-generation sequencing by SPLITSEQ: A new circulating tumor DNA analysis tool

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Introduction

Circulating tumor DNA (ctDNA) analysis requires absolute single molecule sensitivity (Bidard *et al.*, 2013). Recent developments have improved the relative sensitivity of the next-generation sequencing (NGS) at <0.01% (Kinde *et al.*, 2011). However, they still lack absolute sensitivity required to sequence rare templates as low as 10 input mutant molecules. Here we present SPLITSEQ, a novel microfluidics and NGS-based method that combines 0.01% fractional sensitivity with absolute single copy input sensitivity.

Material and Methods

The samples were split into different chambers whereby the input total was limited to 100 molecules, using a highly parallelized microfluidic chip (Access Array, Fluidigm). After Illumina sequencing and demultiplexing, the reads were assigned to the corresponding microfluidic chamber using a double DNA barcoding strategy.

Results and Discussion

We provided multiplexed performance data on mutations in KRAS codons 12 and 13 and BRAF V600E. Using reconstituted DNA solutions from mutant and normal cell lines ranging from 0.01% to 10% mutated allele fractions, we detected the sequence of approximately 0.2, 2, and 20 nominal mutated KRAS and BRAF inputs total in 10 000X background. Single copy numbers were supported by droplet digital PCR (ddPCR) data. Lastly, we confirm the specificity of SPLITSEQ by screening wild-type DNA only and finding a false positive rate significantly lower than ddPCR.

Conclusions

SPLITSEQ detects non-recurrent mutations in tumor suppressors along the length of the sequence and fills an unmet need in ctDNA detection, providing biomarkers from hereto unanalyzable liquid biopsies containing rare mutants.

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Gene Expression In Stromal And Epithelial Laser-Microdissected Prostate Cancer Tissues

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Introduction

Prostate cancer is one the most frequent causes of death for cancer in men in western countries. At present there are no prognostic markers of prostate cancer progression. Metastatic patients are treated with androgen deprivation therapy with scarce efficacy because the disease becomes rapidly androgen-independent. Androgen receptor (AR) is known to be involved in initiation and development of prostate cancer and recently it has been shown to play a different role in the epithelial and stromal compartments of the tumor, acting as tumor suppressor in the former and inducing disease progression in the latter. In this study we aimed at optimizing a protocol for studying gene expression of AR and other markers of tumor progression, such as PSA, EGFR and PTEN, in stromal and epithelial compartments of prostate cancer.

Material and Methods

AR, EGFR, PSA and PTEN mRNA expression was analyzed by qPCR in frozen prostate cancer specimens from 20 patients after laser microdissection of the stromal and epithelial compartments. RPL13a (coding for a ribosomal protein) was chosen as the reference gene. cDNA from samples was preamplified by Taqman Preamp Master Mix (Life Technologies). Preamplification Uniformity was checked on a control sample obtained by extracting RNA from the prostate cancer cell line LNCaP.

Results and Discussion

We successfully set up a preamplification protocol of cDNA involving 20 cycles of preamplification. PSA was more expressed in the epithelial than in the stromal compartment of the tumor, while PTEN presented a higher expression in the stroma. A significant correlation was found between AR and EGFR expression in the epithelial compartment.

Conclusions

Tumor stroma supports prostate cancer progression and several studies show that it may be a promising therapeutic target, therefore it is necessary to optimize methods for investigating the tumor microenvironment. In this context our research provides a contribution demonstrating the possibility of studying gene expression on very limited amounts of input materials such as laser- microdissected prostate cancer tissues.

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Evidence of mRNA shuttled by damaged hepatocytes-derived microvesicles involved in bone marrow cell plasticity

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Introduction

Bone marrow mononuclear cells (BMMC) have demonstrated the ability to differentiate into hepatocyte-like cells under several conditions (Soto-Gutierrez A 2008). Here, we investigated the effect of injured hepatocytes over the differentiation of BMMC's non-adherent and adherent subsets separately, and the presence of specific mRNA shuttled by microvesicles (MVs).

Material and Methods

Bone marrow mononuclear cells (BMMC) and hepatocytes were isolated from adult male Wistar rats (n=12). Mononuclear cells were isolated by density gradient and cultivated for 72h to separate adherent and non-adherent fractions. To induce differentiation process, we employed an *in vitro* co-culture model where healthy and CCl_4-injured hepatocytes were co-cultured with BMMC subsets for 2, 6 and 24 hours. Differentiation was assessed by gene expression of the hepatic genes *Albumin* and C*ytokeratin-18*. Hepatic function was evaluated through measurement of urea in the medium of adherent and non-adherent BMMC after co-culture protocol. Cell-free supernatants from co-cultures were ultracentrifuged at 100,000 g for 1 hour to isolate MVs. RNA was obtained from MVs and we analyzed the presence of *Albumin* mRNA by RT-qPCR.

Results and Discussion

Gene expression analysis showed the presence of hepatocyte markers after 24 hours of co-culture only in the non-adherent subset (differentiated cells). Non-adherent cells showed increase in urea production relative to time of exposure to injured hepatocytes. BMMC subsets co-cultured with healthy hepatocytes did not present signs of differentiation at any point analyzed. Analyzes of the supernatant from differentiated cells revealed the presence of MVs carrying *Albumin* mRNA, suggesting a possible role of MVs in the induction of cell plasticity.

Conclusions

In this study we demonstrate that non-adherent fraction of BMMC express hepatocyte-like characteristics after co-cultured with injured hepatocytes within a short period of time. Our findings suggest that MVs released from the damaged tissue may be one of the mechanisms affecting cell plasticity.

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Quantification of 100 RNA targets from minute samples or single cells

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Introduction

Precise quantification of multiple RNA targets from limiting amounts of starting material is challenging, but often necessary. This is especially true in the developing single cell analysis field. New processes that allow for accurate gene expression profiling from limited samples can benefit researchers in many fields.

Material and Methods

Ntera2 cells, a well-established model system of human stem cell behavior (1), were obtained from the American Type Culture Collection and cultured as directed. To induce differentiation, cells were treated with 10 uM retinoic acid. RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad) and reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad). The cDNA was then processed to enable quantification of 100 target genes. Target gene quantification was by real-time PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and PrimePCR assays (Bio-Rad). For single cell work, cDNA was generated from isolated single cells and processed and analyzed as described above.

Results and Discussion

We have developed a process that quantifies up to 100 RNA targets from minute samples. By analyzing the expression of 100 different RNAs in differentiating NTera2 cells, we find that our process provides accurate and reproducible quantification of both stem cell-specific and neural cell-specific biomarkers. We also used our process to analyze single NTera2 cells. We find that biomarker expression in partially differentiated cells is quite heterogeneous, some cells are like undifferentiated cells, other are like differentiated cells. It is clear that NTera2 cells do not undergo differentiation in a synchronized fashion.

Conclusions

We have developed a process that allows for accurate quantification of up to 100 target genes from very small samples including single cells. We envision that our process can benefit researchers who work with limited or rare samples, and can lead to advances in the single cell analysis field.

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From single cell MALDI-TOF profiling to protein identification in bovine oocyte: integrative approach to study oocyte maturation

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Introduction

In mammals, developmental potential of female germ cell, an oocyte, is acquired during pre-conceptual period and especially throughout oocyte meiotic maturation. A number of proteins are produced in the oocyte during maturation to assure fertilization and early embryo development. Bovine oocyte is 100-130 microns in diameter and contain about 80 ng of proteins; thus the use of classic differential proteomics approaches is limited by the scarcity of biological samples.

Material and Methods

Bovine immature oocytes were collected from slaughterhouse ovaries and then subjected to in vitro maturation (IVM) or recovered in vivo by ovum pick-up after ovarian stimulation. Intact Cells Matrix-Assisted Laser Desorption/Ionisation time-of-flight Mass Spectrometry (ICM-MS) profiling was applied for analysis of single oocyte. Bottom-up and top-down proteomic approaches using high resolution nano-LC/MSMS on the pools of the immature and IVM oocytes (n=200 per group) were used for proteins identification and quantification by spectral counting (Scaffold 3G+).

Results and Discussion

ICM-MS approach was adapted to single bovine oocyte by using MALDI-TOF mass spectrometer operating in positive linear mode. Therefore we obtained and compared the spectra profiles as fingerprints of different oocytes. Objective was to look for differential proteins between the oocytes that were able (mature) or not (immature) to be fertilized. Reproducible mass fingerprint gathering a number of peaks ranged 3 000 – 25 000 m/z were obtained for groups of immature and mature oocytes (n=30). Intensity peak variations were observed for 40 from 114 of detected peaks between the oocytes of two groups (ANOVA, $p<0.01$, Progenesis-MALDI, Non-Linear Dynamics). Among them, intensities of 14 peaks varied more than two fold. Using bottom-up approach, 299 different bovine proteins were identified. Among them, 163 proteins were more abundant and 12 proteins were less abundant in mature oocytes as compared to immature ones ($p<0.05$). Also, several proteins corresponding to m/z peaks (<10 kDa) were identified by Top-down approach.

Conclusions

Single cell MALDI-MS was adapted to bovine oocyte and allowed the discrimination of the oocytes in different maturation stages by protein profiling of a number of small size proteins (3-25kDa). Bottom up and top-down approaches were applied for the identification of the differential proteins between immature and mature oocytes.

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Single cell RNA-seq for the study of rare endothelial cell subpopulations

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Introduction

Vascular angiogenesis and endothelial cell differentiation are important characteristics of development and are required for overall homeostasis. In contrast, vascular angiogenesis in tumors is considered to be detrimental as resulting blood vessels provide nourishment to the dividing tumor cells. However, recent studies have shown that not all vascular endothelial cells are damaging and in fact, certain types have a favorable result on prognosis. We are interested in understanding the molecular mechanisms that drive blood endothelial cell differentiation under both homeostatic conditions and during tumor development.

Material and Methods

Murine blood endothelial cells were sorted by FACS using well known cell surface markers. With the Fluidigm C1 system, we used fluorescence microscopy to confirm the identity of each single cell in the C1 chip. After cDNA preparation with the Clontech SMARTer technology, we further confirmed cell identities by RT-qPCR of known endothelial-specific genes. We also verified they were negative for hematopoietic cell genes. We used the Nextera XT system and Illumina HiSeq2000 for library preparation and sequencing.

Results and Discussion

Due to the low frequency of our cells of interest and the difficulty of their isolation from tissues, this method allowed us to verify each single cell using various techniques - FACS, fluorescence microscopy and RT-qPCR. Extensive pre-verification made it possible to characterize each individual cell before sequencing and compare it to the data later acquired by the RNA-seq. We also ensured that there were no contaminating hematopoietic cells that may interfere with data analysis.

Conclusions

Here we show that single cell RNA-seq is a viable option for the analysis of low frequency cells in combination with good sorting and verification methods.

Performed in close collaboration with the Génome et Transcriptome (GeT) Platforme, Toulouse

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