

Intracellular Gene Expression Profiles Revealed with Real-time PCR Tomography



Dr. Mikael Kubista
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Application:

- Gene Expression

Fluidigm Technology:

- BioMark System
- 48.48 dynamic array

Dr. Mikael Kubista, considered to be a pioneer in the advancement of real-time polymerase chain reaction science, has also developed a novel way to explore the genetic makeup of single cells.

In a research paper entitled: *Intracellular expression profiles measured by real-time PCR tomography in the Xenopus laevis oocyte*, Dr. Kubista and fellow researchers describe a new method for measuring localized RNA expression profiles within single cells. The method is demonstrated by using real-time PCR tomography to slice the *Xenopus laevis* (African Clawed Frog) oocyte into thin layers and then profile the slices.

The BioMark system enabled measuring differentiation on the cell level with high accuracy and throughput.

systems. The main systems are early development [of embryos], and the oocyte. We work with *Xenopus Laevis*. The advantage is that

the frog's oocyte is very large. That allows us to do the qPCR tomography and measure the intracellular gradients. We do high-resolution profiling of the mRNA gradients within the cell. We are collecting samples at early stages, two-cell, four-cell stages, and so forth, separating cells and seeing whether the cells are the same or not. Do they differ essentially? When is the heterogeneity introduced into the developing [embryo]? What kind of profiles or gradients are formed? The amphibian development is different than human. You have all the mRNA needed for several cell divisions in the oocyte. When the cell divides the RNA is divided too, without new being synthesized for several divisions. That makes it a very interesting model system."

Advances in research technologies and protocols in recent years have enabled this sort of research, he explained.

"This has not been possible before, because in science we had not been able to measure the differentiation on the cell level, at least not with high accuracy," he said. "Using Fluidigm's integrated fluidic circuit technology, measuring

Dr. Kubista is the head of the Gene Expression Laboratory at the Institute of BioTechnology and the founder of the TATAA Biocenter in Sweden.

"Our main interest is single cell expression profiling," Dr. Kubista said. "We're looking into quite a few different



the cell differentiation is becoming possible today. We are doing it on Fluidigm's BioMark system, using 50 genes per cell and using preamplification. If you measure 50 genes of 200 cells, well obviously you'll have ten thousand data points there and you need high throughput.

When we pioneered this field with our first paper in 2005, we measured five genes per cell. In those days we didn't even have a preamplification protocol. There are actually two things that are important: A robust preamplification protocol and a high-throughput system."

Secondarily, the researchers found that the frog oocyte's two classes of mRNA, animal and vegetal, are polarized along an axis. Most of the animal mRNA is located at one end, while most of the vegetal mRNA is located at the other end of the axis.

Thirdly, the researchers reported that mRNA expression profiles did not change following *in vitro* fertilization of the frog oocyte.

The Research Experiment

The research paper outlines the frog oocyte research experiment as follows:

Four types of eggs were collected: unfertilized eggs and eggs collected at 25, 50 and 85 minutes post IVE. The eggs were frozen at -70°C. The eggs were embedded with optimum cutting temperature compound and dissected into 35 slices across the A-V axis. Consecutive slices were pooled into five groups with seven slices in each. From each group, 200-500 ng of total RNA was extracted

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using RNeasy Micro kit (Qiagen). RNA concentrations were determined with the Nanodrop® ND1000 quantification system and RNA quality was assessed with the 2100 Bioanalyzer using the RNA Pico Chip (Agilent). Total RNA was reverse transcribed (High Capacity cDNA Archive Kit -Applied Biosystems). PCR was run in an Mx3005P (Stratagene). Gene expression data were analyzed using GenEx software from MultiD Analysis and Prism4 from Graphpad.

The conventional real-time PCR results were confirmed for selected genes with digital PCR using the BioMark Digital Array™ IFC. The array is designed to accept 12 sample mixtures, which each is partitioned into a different 765-chamber grid. One-step RT-qPCR was performed directly on the chip. Ten microliter reaction mix was loaded onto the chip. The input amount of total RNA was tuned to produce less cDNA molecules than the number of chambers. The mixture was distributed into 765 chambers and then analyzed by PCR. FAM/ROX fluorescence signal was collected at the end of each cycle, and the number of chambers that gave positive fluorescence signal after 40 cycles was registered. The number of mRNA molecules in the sample was grossly estimated assuming 80% cDNA synthesis

Reagents and Equipment

- BioMark™ RT-PCR system
- Fluidigm® 48.48 Dynamic Array™ IFC
- RNeasy Micro Kit (Qiagen)
- Nanodrop ND1000 quantification system
- 2100 Bioanalyzer
- RNA Pico Chip (Agilent)
- High Capacity cDNA archive kit (Applied Biosystems)
- MultiScribeReverse Transcriptase
- SureStart Taq DNA Polymerase (Stratagene-Europe)
- SYBR Green (Molecular Probes)
- MX3005P (Stratagene-Europe)
- GenEx Software (MultiD Analysis)
- Prism 4 (Graph Pad)
- Superscript RT/Taq (Cells Direct qPCR-RT Kit, Invitrogen)

yield in the reverse transcription reaction.

The Results

Out of 18 genes studied, 11 were found to be located at the animal pole and seven at the vegetal pole. The genes at the animal pole included: FoxH1, Oct60, GSK-3β, EF-1α, Xmam, Tcf-3, GAPDH, β-catenin, XPar-1 and Stat3. Genes at the vegetal pole included: VegT, Vg1, Xdazl, Wnt11, Otx1, Dead-south and Xcad2.

"In summary, real-time PCR tomography can measure intracellular mRNA gradients more sensitively and with greater resolution than traditional *in situ* hybridization," Dr. Kubista and his colleagues concluded in their paper.

As for other uses of the PCR tomography technique, the researchers suggested the following: "the localization of nuclei through genomic DNA, of mitochondria through mitochondrial DNA and of translationally active sites through ribosomal RNA. The tech-

niques can also be used to localize viruses and bacteria in tissue sections.”

Other Related Research

Dr. Kubista said his frog oocyte research relates to other work he does with stem cells and circulating tumor cells (CTC).

“We’re trying to learn how the asymmetry is introduced during [embryo] development and trying to understand the differentiation process of the cells,” Dr. Kubista said. “What actually makes tumor cells or dormant cells to be different from the original cell or mother cell? This is what we are interested in. We are collecting

“A seemingly homogeneous population of cells—either from tissue or even in the cell culture—in fact, show quite large variability in expression.”

stem cells—either embryonic stem cells or neural progenitor cells—we are sampling individual cells and doing gene expression profiling,” he said. “We know these are at least initially the same cell type, however, using Fluidigm’s technology, we are detecting that there are actually a number of subtypes of these cells that can be identified by expression profil-

ing. So, a seemingly homogeneous population of cells—either from tissue or even in the cell culture—in fact, show quite large variability in expression. What we do not know is whether these expression variations are reversible or not. Are there really different populations that cannot switch back? Or, are they different phenotypes of the same population?”

Identifying Stem Cells among Tumor Cells

“One of the most exciting things we are doing in this field now is collecting circulating tumor cells from cancer patients,” he said. “This has been around for a while, but in practice you are only counting circulating tumor cells that the patient has in the blood, and depending on the count, you, for example, assess the effect of the therapy, and whether the individual will relapse.”

Dr. Kubista and his team go a step further in their research.

“We are also studying cells by expression profiling,” he said. “We have a panel of 40 genes, which are tumor and stem cell markers. We hope that this will be a very valuable indicator of whether an individual has a high risk to develop metastasis, for example. We know today that the individuals have thousands of circulating cancer cells, but most of them are actually not dangerous because they are simply shredded from the primary tumor. However, we know that very few of them may be the critical ones in a particular cancer, these are cancer stem cells. So what we are really trying to do is to identify the stem cells among the circulating tumor cells.”

Quality Consortium Formed

Dr. Kubista is very dedicated to improving quality in genetic research and is involved in a new project to improve sample management.

“This is a area where we think that Europe is ahead of the US,” he said. “We have established a pan-Europe project for sample management. Our goal is to improve the process from collecting the sample and handling it prior to actually doing the qPCR. Today, qPCR is highly robust; it’s not where the uncertainty comes from in research. Uncertainty comes from the collection of the samples. Today, if I take a blood sample from you, 10 seconds later, the gene expression has changed. It’s not reflecting the blood that was in you any more, it’s the blood that’s in my collection tube. We need methods to control this, we need protocols and standard operating procedures. So there’s a major consortium in Europe to tackle this, it’s called SPIDIA (www.spidia.eu). The consortium is headed by Qiagen and the European Committee for Standardisation, CEN, is involved. It’s information sharing, defining protocols, finding procedures to find markers to test the quality of the samples. We’re looking at the preparation of RNA, DNA, proteins and metabolites from blood and nucleic acids and proteins from tissue. For this particular work, the Fluidigm BioMark is useful, because we are screening all those samples to find markers that are affected during the storage process or suitable markers for quality control of those samples. So we are not looking for disease markers, but we are looking for markers for quality.”

TATAA Offers Hands-on Real-time PCR Classes

The TATAA Biocenter, where Dr. Kubista teaches, offers real-time PCR services and hands-on training course in Scandinavia, Central Europe and the U.S.

In the expression profiling course they use a BioMark system with 48.48 Dynamic Array™ IFCs and compare it with 96-well plate systems in the class hands-on experiments.

“We have 12 students, one group running the BioMark and the others are running four regular 96-well plate instruments and they’re running them sequentially,” he said. “The 96-well plates take much, much more time than running the BioMark. At the end of the day the students using 96-well plates only have this much data [gestures an inch], and the BioMark students have this amount of data [gestures a

foot]. On their faces you can see the difference. If you are doing expression profiling, in my opinion, this requires at least 20 genes. In theory you can do it with two genes, but realistically,

“We see a 25-fold time difference between the BioMark and traditional 96-well plates in our class experiments.”

20 genes from a minimum of 20-30 samples is required. That’s a lot of work with 96-well plates. We see a 25-fold time difference between the BioMark and traditional 96-well plates in our class experiments.”

Sometimes less is more, cautions Dr. Kubista when it comes to selecting genes for study.

“A big mistake a lot of groups are making is running too many genes,” he said. “If you run genes that are not sensitive to whatever you are studying, your data quality actually decreases. Ninety-nine percent of genes in the complete genome are not informative for a given condition. They just make noise. And perhaps we’ll get to the point where we have 96 genes to study, but for most purposes, in my mind, between 20-50 is realistic. But you have to identify them. The way to identify them is to start with a large number of genes and select the most informative ones.”

TATAA organizes the main US qPCR symposium in Millbrae, Calif. (www.qpcrsymposium.com) November 9-12, 2009. “We will present some new data on qPCR experimental design,” he said.

Biography

Professor Mikael Kubista was among the pioneers who developed real-time PCR. Starting in 1991 his laboratory developed dyes and probes for real-time PCR and in 1998 they founded LightUp Technologies (www.lightup.se) as one of the first companies focusing on real-time PCR based human infectious disease testing. His team then developed experimental approaches for accurate measurements of expression levels by real-time PCR, and they pioneered the fields of single cell expression profiling and multiway expression. Kubista also developed methods and approaches to analyze gene expression data and founded the company MultiD Analyses (www.multid.se) that develops the popular software GenEx for real-time PCR data analysis. Working as advisor for Unesco, he introduced real-time PCR in Africa and in the Middle East. In 2001 Kubista founded the TATAA Biocenters (www.tataa.com), as the world leading service providers and organizers of hands-on training in real-time PCR. Annually, TATAA Biocenter arranges the main QPCR symposium in US (www.qpcrsymposium.com). Most recently, Kubista set up the first high throughput qPCR expression profiling center in Europe at the new institute of Biotechnology in Prague (www.ibt.cas.cz).

References and Related Links

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