ScientistSpotlight

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Enter a new dimension of single-cell analysis

Introduction

With the development of a method that allows for detection of RNA by flow cytometry, researchers have a tool to unmask previously unanswered questions. Dr. Filippos Porichis PhD of The Ragon Institute of MGH, MIT, and Harvard, has been at the cutting edge of the technology's adoption. In a recent *Nature Communications'*[1] paper, Dr. Porichis showed the simultaneous detection of microRNA, mRNAs, and proteins at the single-cell level, highlighting the ability of this method to be used as an invaluable tool for investigating the molecular networks of any cell of interest.

Affymetrix: What is the goal of the Ragon Institute?

Porichis: The Ragon Institute of MGH, MIT, and Harvard was established in 2009 with a gift from the Philip T. and Susan M. Ragon Foundation, creating a collaborative scientific mission among these institutions to harness the immune system to combat and cure human diseases. Under the leadership of Dr. Bruce Walker, the initial focus of the institute is to contribute to the development of an effective AIDS vaccine. The Ragon Institute draws scientists and engineers from diverse backgrounds and areas of expertise across the Harvard and MIT communities and throughout the world to apply the full arsenal of scientific knowledge to understanding mechanisms of immune control and immune failure and apply these advances to directly benefit patients.

The Ragon Institute has maintained an active involvement and presence in Africa since the early 1990s. The Institute is committed not only to providing scientific expertise to a continent devastated by AIDS, but also to providing HIV education and support to persons infected and affected by HIV/AIDS.

Affymetrix: What is the focus of your research?

Porichis: Our research group at the Ragon Institute focuses on the immunological aspects of T-cell function in HIV and Tuberculosis (TB) infections, and the implementation of novel techniques in order to evaluate the immunoregulatory networks that lead to T-cell dysfunction. Immunotherapeutic interventions targeting inhibitory pathways to restore T-cell function is an emerging field, and the first drug targeting the PD-1 pathway was recently approved by the FDA to treat melanoma cancer. Such an approach is also considered in chronic infections, such as HIV and TB. During the last six years, I have investigated the immunoregulatory networks that govern HIV-specific T-cell dysfunction and have showed that manipulation of inhibitory pathways, such as PD-1 and IL-10, can restore function of pathogen-specific T-cell responses. We also showed that HIV-specific CD4 T-cells are regulated by different patterns of inhibitory receptor other than HIV-specific CD8 T-cells.

The majority of current antiretroviral drugs target the viral cycle and are not designed to directly improve the immune system. Our data showed that blockage of the PD-1 pathway can restore effector function of HIV-specific CD4 T-cells, even in subjects with controlled viremia on antiretroviral treatment, suggesting that

Dr. Filippos Porichis, PhD

Dr. Porichis is the Director of International Programs at the Ragon Institute and serves as a member of the Executive Committee of the MGH Center for Global Health. He is an Assistant in Immunology at Massachusetts General Hospital and an Instructor in Medicine at Harvard Medical School. Originally trained in Biomedical Sciences (BSc and MSc) at the University of Portsmouth, UK, he received his PhD from the University of Crete in Greece and the Institute of Molecular Biology and Biotechnology (IMBB) and subsequently joined the Ragon Institute for his postdoctoral training under the supervision of Dr. Daniel Kaufmann.

"FlowRNA enables the detection of nucleic acid sequences at the single-cell level without being limited by antibody availability, therefore allowing the detection of many more markers that remained undetected with conventional antibody-based flow cytometry."

immunotherapeutic interventions can complement current ARV treatment to restore T-cell function. Finally, through collaborative studies we identified the epigenetic regulation of PD-1 expression, as well as the transcriptional signatures of T-cell exhaustion downstream of PD-1 in HIV-specific CD8 T-cells, and showed that knockdown of a transcription factor BATF can restore function of CD8 T-cells.

Currently we are working on understanding the functional characteristics of HIV-specific CD4 T-cells that prime effective humoral responses for the development of broadly neutralizing antibodies against HIV. There is strong evidence that an effective prophylactic or therapeutic HIV vaccine will require potent HIV-specific CD4 T-cell responses to provide help to both cellular and humoral arms of the immune system. Therefore, understanding the properties of effective CD4 help that contributes to protection through interaction with antigen presenting cells, CD8 T-cells, and B cells, is critical for the rational design of vaccine strategies.

Affymetrix: When did you start using Affymetrix' RNA fluorescent *in situ* hybridization (FISH) and branched-DNA amplification technology to investigate single-cell gene expression?

Porichis: We started working on this assay in July 2010, while I was a Postdoctoral Trainee under the supervision of Dr. Daniel Kaufmann (who recently moved to the University of Montreal). At that time, the company called Panomics, later acquired by Affymetrix, had developed a FISH assay for microscopy. In collaboration with Dr. Daniel Kavanagh at the Ragon Institute, we tried to implement this assay to investigate HIV interaction with immune cells. However, given our strong immunology background in the Kaufmann lab, we found microscopy very cumbersome and therefore decided to adopt this assay for flow cytometry. In August 2010, I used the FISH assay to detect RNA expression with flow cytometry for the first time, but the buffers at that time were not optimized for such a procedure. Panomics was then acquired by Affymetrix, and with the new infrastructure and technological breakthroughs, the company was able to optimize the assay to produce the first-generation FlowRNA assay launched in May 2013.

Affymetrix: What drove you to use this technology with flow cytometry? What is the benefit of using RNA FISH, branched-DNA amplification technology with flow cytometry?

Porichis: Single-cell analysis is a rapidly growing field in biomedical research that yields an unprecedented level of information. Flow cytometry is the gold standard for high-throughput phenotypic and functional characterization of various cell types. While the major breakthrough in flow cytometry was the use of fluorescently labeled antibodies for the detection of intracellular or surface markers, dependence on antibodies is also its major constraint due to the limited availability of highly specific and sensitive reagents for antigens.

FlowRNA enables the detection of nucleic acid sequences at the single-cell level without being limited by antibody availability, therefore allowing for the detection of many more markers that remained undetected with conventional antibody-based flow cytometry. FlowRNA also has great applicability in animal species studies limited by antibody availability.

Affymetrix: If FlowRNA was not available, what technologies would you have used in your research, and what limitations would that create?

Porichis: Single-cell transcriptomic analysis is now possible, and several studies have been able to use this approach to uncover the heterogeneity of seemingly homogeneous populations. However, such approaches are still in development, quite expensive, labor intensive, and require the use of specific instruments and technical expertise.

"Flow cytometric identification of RNA species has several major comparative advantages because it is high-throughput and does not require single-cell sorting (which adds extra steps of cell loss and stress), so one can use fewer cells to yield maximum data output."

Additionally, FlowRNA can simultaneously detect mRNA and protein expression, and therefore can be used to mechanistically and phenotypically characterize co-expression of RNAs with the functional proteins at the single-cell level.

Finally, the user-friendly protocol that has many similarities with standard antibody-staining procedures for regular flow cytometry, and the processing and data acquisition with standard laboratory equipment and a cytometer, make this assay an invaluable tool for any immunology lab performing translational research.

Compared to FISH microscopy, which is practically limited to the analysis of a few hundred cells, flow cytometry allows high-throughput acquisition of millions of cells in a single sample, providing a larger variety of fluorescent channels, such as 10 to 15 fluorescent markers on four- or five-laser cytometers.

Affymetrix: With the ability to correlate genomic and protein data within a single cell, what does this mean for the future of cellular dynamics research?

Porichis: In a study recently accepted in *Nature Communications,* we showed the simultaneous detection of microRNA (miRNA), mRNAs, and proteins at the single-cell level, highlighting the ability of this assay to be used as an invaluable tool for investigating the molecular networks of any cell of interest.

Using this assay, one can perform in-depth analysis of miRNA function in combination with their translational (mRNA level) and expressional (protein level) effects in both normal and pathologic conditions. In the same manuscript we also showed that the FlowRNA assay can be used for the high-quality mRNA detection using the Imagestream technology, so one can perform high-throughput comprehensive visual analysis of RNA-protein interaction at the single-cell level.

Affymetrix: Specific to your future research, where do you see this technology being applied?

Porichis: One of the challenges of this novel assay is the conceptual understanding that detection of mRNA relies on different biologic processes than proteins, and therefore the standard *in vitro* immunologic assays performed with flow cytometry, such as intracellular cytokine staining, have completely different biologic backgrounds defined by different kinetics, so detection requires distinct experimental designs. This may be trivial for molecular biologists, but for immunologists who have been primarily using protein-based flow-cytometric assays (ICS), this may prove an unforeseen obstacle that can hold back their initial efforts to adopt this assay. In the study published in *Nature Communications,* we have optimized mRNA detection of cytokines from HIV-specific T-cells after stimulation with their cognate antigens, and address the differences observed between regular protein detection with ICS, and mRNA detection with the FlowRNA assay.

"FlowRNA is now an integral part of the immunologic assays in our laboratory at the Ragon Institute to evaluate HIV-specific T-cell function. It enables the detection of transcription factors and intracellular regulators for which we previously had no means of detection apart from qRT-PCR assays on bulk cells."

As this assay continues to develop, we are constantly trying to optimize detection for novel markers, while at the same time trying to minimize background staining issues. Our ability to characterize immune responses at the transcriptional and translational level has increased significantly.

Reference:
Porichis F, Hart MG, Griesbeck M, Everett HL, Hassan M, Baxter AE, Lindqvist M, Miller SM, Soghoian DZ, Kavanagh DG, Reynolds S, Norris B, Mordecai SK, Nguyen Q, Lai C, Kaufmann DE., High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry. Nature Communications **5**:5641 (2014). doi:10.1038/ncomms6641.

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Affymetrix, Inc. Tel: +1-888-362-2447 ■ **Affymetrix UK Ltd.** Tel: +44-(0)-1628-552550 ■ **Affymetrix Japan K.K.** Tel: +81-(0)3-6430-4020
Panomics Solutions Tel: +1-877-726-6642 panomics.affymetrix.com ■ **USB Products** Tel: +1-800-321-9322 usb.affymetrix.com

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