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MEGA MULTIPLEXING FOR IMMUNOHISTOCHEMISTRY

Francesca Bosisio and Frederik De Smet specialize in performing a high-plex mIHC method that uses conventional antibodies and pathologist-driven data analysis.

Interviewed by Niki Spahich, PhD

Above image: Courtesy of Rob Stevens, KU Leuven

An appreciation of tissue complexity in disorders such as cancer has led researchers to develop multiplex immunohistochemistry methods (mIHC), where dozens of markers are assayed on a single slide. These methods lend insights into disease states by highlighting tissue architecture at the single-cell level within spatial context.

Dermatopathologist Francesca Bosisio and bioengineer Frederik De Smet are co-chairs of the KU Leuven facility for mIHC. Their flagship high-plex mIHC technology, Multiple Iterative Labeling by Antibody Neodeposition (MILAN), is a cyclic staining method that uses conventional fluorescent antibodies to stain up to 80 markers on the same slide.[1] In their facility, they offer end-to-end mIHC services for scientists at KU Leuven and beyond. In this interview with *The Scientist*, Bosisio and De Smet discuss MILAN's origin and the benefits of mIHC.

Q. How was MILAN developed?

Francesca Bosisio: When I was a medical resident at the University of Milano-Bicocca, I was working with Giorgio Cattoretti, and we thought it was time to have more than ten markers on a pathology slide. To develop an mIHC protocol, we started comparing different antibody stripping methods. We spent quite some time developing an efficient stripping solution that was gentle on tissue.

Then I came to KU Leuven for graduate school, and the staining method was ready to be tested. I had a panel of 39 markers to investigate the microenvironment in primary melanoma tissues. Suddenly, I had a huge number of multiplex images with no way to analyze them. It took us two years to develop our own pipeline from scratch, to go from the single cell signals to drawing inferences about cellular relationships in the tissue. That led us to develop our own analysis software and to understand how the activation status of T lymphocytes changes in different regions of primary melanomas.[2]

Q. How does MILAN compare to other mIHC methods?

Frederik De Smet: Most other technologies are confined to a one centimeter or smaller area. With MILAN, we analyze the entire surface of a regular histology slide.

FB: We do not use engineered antibodies; we can use any antibody of interest for the project after we have validated it. If a researcher has developed their own antibody, we can implement it immediately. Additionally, MILAN uses secondary antibodies to amplify signals. This allows the capture of lower intensity signals, so the range of a protein's expression that can be detected is broader than when using primary antibodies without an amplification system.

Q. How does your facility optimize the MILAN method?

FDS: We curate the whole process from start to finish. When researchers bring their samples to our facility, we have pathologists involved. It is a challenge to integrate the spatial and single cell data. It is important to have somebody with years of knowledge and training looking at the stained tissues. Pathologists can determine if certain signals are relevant before a researcher dives into the interactions they identified.

Also, we have in-house instrumentation that can do a lot of the work automatically, which is beneficial for standardization and data quality. The data analysis is usually challenging, so we are working on a solution where someone without bioinformatics skills can understand the expression levels that are present in different phenotypes.

Q. How does mIHC benefit cancer research?

FB: Cancer has a complex immune ecosystem. There are different types of inflammatory cells fighting the tumor, and the tumor itself is heterogenous, assuming different cell states. To characterize every cell, researchers need to stain specific markers in each of these cancer cells. This way we can show a complex picture for each patient in terms of their specific cancer and the inflammatory population composition. If we can see the patient-specific heterogeneity, we can find a better drug that addresses the patient's particular cancer.

In a recent study, we mapped the immune phenotypes of patients with melanoma before receiving immunotherapy.[3] The spatial component of mIHC is what made the difference in our results. To determine if a patient would respond to immunotherapy, it was important to see the expression of markers on certain cell types that were located in a specific part of the tumor.

FDS: For glioblastoma, the currently available immunotherapy approaches are not working well. To explore new strategies, we need to know where certain cells are located, how are they interacting, and the tissue architecture. We are using mIHC to understand a spectrum of patient profiles and determine how we could intervene in a personalized way.

This interview has been condensed and edited for clarity.

qPCR: DRIVING WASTEWATER SURVEILLANCE FOR INFECTIOUS DISEASE

Natalie Knox and the Public Health Agency of Canada's National Microbiology Laboratory are helping establish a national qPCR-driven wastewater surveillance network for SARS-CoV-2 and other diseases.

Interviewed by Nathan Ni, PhD

Above image: Courtesy of National Microbiology Laboratory, Public Health Agency of Canada/ Natalie Knox

SARS-CoV-2 is shed in the fecal matter of infected individuals and, therefore, can be detected in wastewater using quantitative polymerase chain reaction (qPCR). Scientists used qPCR-driven wastewater surveillance throughout the COVID-19 pandemic as a means of gauging viral prevalence—including the presence and distribution of variants of concern—within communities, avoiding the need for blanket mass testing.[1,2]

The Public Health Agency of Canada's National Microbiology Laboratory (NML) established a wastewater surveillance network spanning the nation's three coasts.[3-6] In an interview with *The Scientist*, Natalie Knox, director of One Health at the NML, discusses these efforts to safeguard Canadians against current and future threats.

Q. Why is wastewater surveillance important and how has qPCR become the gold standard technique?

We can get a lot of valuable information from a single wastewater sample that lets us determine trends and measure the burden of disease within a community. qPCR has become the gold standard because it is a relatively simple technology that has been established and tested over several decades. It is cost effective and does not require a lot of equipment, so any standard molecular laboratory—public health, academic, or even third-party private labs—can perform wastewater testing.

However, wastewater testing is more complex than clinical sample testing. Wastewater is highly variable. It is influenced by things such as industrial discharge, recent climate patterns (i.e. snow melt), and large precipitation events, which affects how qPCR test results are interpreted. There are some quality control indicators that we can use, but no single indicator works for every single wastewater infrastructure setup. A method that yields great results in one treatment plant may not work very well in another one. Choosing which indicators to apply to specific wastewater infrastructure situations is something that needs careful evaluation, and there is a lot of research being done in this space right now.

Q. How translatable are COVID-19 qPCR workflows for other infectious diseases?

For some diseases, good clinical targets already exist that scientists can optimize for wastewater testing. In other cases, additional validation and optimization is needed. Also, a rapidly evolving pathogen such as influenza virus can manifest mutations in primer-target regions, which can lead to erroneous results. We have to constantly monitor our assays to ensure that they are operating as expected.

Q. Why is qPCR data good for disease modeling?

The purpose of modeling is to give us a short-term forecast so that we can make plans accordingly. For example, the remote northern Canadian communities that we work with use the wastewater signal to evaluate risk to their community members. There was one instance where the increasing wastewater signal helped them adopt protective measures for a public community event where there was the potential for a lot of exposures.

At the beginning of the pandemic, many different data sources were available for modeling. As the pandemic progressed, data became less available, to the point where wastewater is the primary source for models right now. This is a trend that will likely continue because clinical testing right now is no longer representative of the population.

Q. How can researchers use wastewater surveillance to track endemic diseases?

We know that respiratory syncytial virus (RSV) and influenza virus have seasonal patterns. We would like to use this information to denote the start of the flu or RSV season in coordination with vaccination campaigns and appropriate prophylactic treatments for at-risk populations. This is where wastewater testing can help inform specific public health actions to ensure that those communities and vulnerable populations are protected.

Q. Finally, what are the objectives for your team going forward?

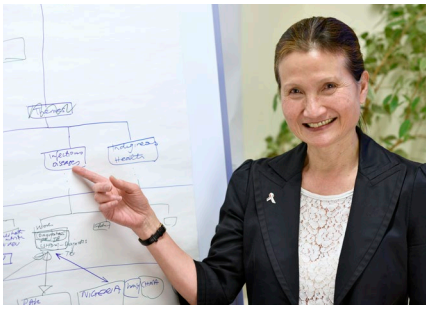
The biggest challenge right now is determining the sustainability of a wastewater program across Canada. That involves figuring out which sites to sample from, making sure testing can be accessed fairly, and determining ideal sampling frequency. It also involves setting clear thresholds and interpretation guidelines for public health. We have spent a lot of time developing methodologies over the last three years, and we have reached the point where we need to be able to take action based on the data.

This interview has been condensed and edited for clarity.

ACCESSIBLE LATERAL FLOW ASSAYS: TEST TO TREAT, TEST TO PROTECT

From development to implementation, Rosanna Peeling discusses the importance of rapid tests for public health.

Interviewed by Deanna MacNeil, PhD



Above image: Courtesy of Rosanna Peeling

Scientists and public health experts built the COVID-19 pandemic response on years of experience navigating infectious disease outbreaks. Although lateral flow immunoassays have been central to clinical diagnostics for over six decades, their public health use during the pandemic cast a new spotlight on this veteran testing technology.[1]

Medical microbiologist Rosanna Peeling is no stranger to progress in diagnostic testing. After establishing a national screening program for chlamydia infections in Canada, she led the Health Canada National Laboratory for Sexually Transmitted Diseases. Peeling then joined the World Health Organization (WHO) as Head of Diagnostics Research in Tropical Diseases, where she coined the concept of ASSURED diagnostics. The tenants of ASSURED embody three key ideals in diagnostic approaches—accuracy, affordability, and accessibility.[2] At the London School of Hygiene and Tropical Medicine (LSHTM) and International Diagnostics Centre (IDC), her current research focuses on test development, evaluation, and implementation in developing countries.

In an interview with *The Scientist*, Peeling explained how lateral flow antigen tests emerged as a solution to accessible and affordable diagnostics for sexually transmitted infections, highlighting how self-testing became the star of the COVID-19 public health response, and her wish list for rapid tests in the future.

Q. What tests were initially involved in infectious disease diagnostics?

For chlamydia, at first scientists cultured the bacteria in the laboratory, which took at least 72 hours. This strategy was not very good because by the time you transported the specimen back to the laboratory from the clinic, the bug may have died. We needed a method that would not require culture that was accessible to primary health care physicians, clinics around the city, or remote areas. In the 1990s, scientists started using molecular tests, including PCR and antigen tests that were not quite like today's lateral flow assays. These tests still required instrumentation, but they were much more accessible. To increase the sensitivity and specificity of these assays, we needed molecular tests that detected DNA, but those were not accessible financially or physically at the time. For laboratory-based immunoassays, scientists purify the antigen from the sample and incubate it in a well for 30 minutes to an hour. In contrast, the reaction in a typical lateral flow assay takes only seconds. We gradually progressed to lateral flow assays, which are accessible and affordable, but not as sensitive as laboratory-based immunoassays.

Q. Aside from the COVID-19 pandemic, are there other examples of lateral flow self-testing that have contributed to infectious disease diagnostics?

Rapid tests for HIV were among the first lateral flow assays for self-testing. The Federal Drug Administration (FDA) first approved HIV self-testing in 2012 based on modeling by the Centers for Disease Control and Prevention (CDC), which showed that self-tests at a pharmacy or at home would help countries achieve the famous 90-90-90 target set by the United Nations and WHO. The goal is for 90 percent of those who are infected to know their status through testing, 90 percent of those who test positive to have access to treatment, and 90 percent of those who are treated to become viral-negative or controlled. For HIV, the implications of testing positive are still enormous. Even with treatment, there is stigma and uncertainty. People like having the option to test themselves in the convenience of their own homes without going to a testing center.

Q. What made at-home testing so successful as a COVID-19 public health response?

Diagnostics are not only for case detection. The threshold of SARS-CoV-2 virus transmission is about one million viruses per specimen from the nasal passage. If a patient has a viral load below that, the chance of transmission is low. COVID-19 lateral flow tests detect up to roughly one million viruses per sample, so they detect the people with the highest risk of transmission. These tests took on a new and important public health role to interrupt the chain of transmission within communities.

Q. What future do you envision for self-testing with lateral flow assays?

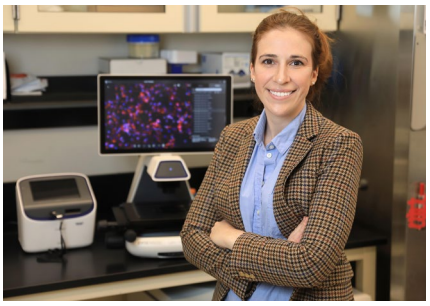
I think simple rapid tests that allow clinicians and families to know whether an infection is bacterial or viral would be ideal. We would like to develop tests that reduce unnecessary prescriptions for antibiotics, and tests for diseases of epidemic potential that we want to detect early, such as respiratory syncytial virus (RSV), influenza, and coronaviruses. That is the biggest wish list right now.

This interview has been condensed and edited for clarity.

SPECTRAL FLOW CYTOMETRY: SEPARATING THE FLUOROPHORE RAINBOW

Spectral flow cytometry separates similarly emitting fluorophores and enabled Kaitlyn Sadtler to construct a 24-color rat panel for immunological analyses.

Interviewed by Charlene Lancaster, PhD



Above image: Courtesy of NIBIB/Chia-Chi "Charlie" Chang

Flow cytometry is a widely used analytical technique that distinguishes between cell populations depending on the presence or absence of chosen markers. However, the number of markers that can be included in a flow cytometry panel is limited by the number of fluorophores with distinguishable emission spectra. Spectral flow cytometry, an evolved iteration of conventional flow cytometry, overcomes this problem by differentiating between similarly emitting fluorophores, thereby increasing panel sizes.

Kaitlyn Sadtler, an Earl Stadtman Investigator and Chief of the Section for Immunoengineering at the National Institute of Biomedical Imaging and Bioengineering (NIBIB), studies the immune response to tissue trauma and medical device implantation. In a recently published *Cell Tissue Organs* paper, Sadtler's laboratory developed a 24-color, spectral flow cytometry panel to investigate immune cell infiltration at the site of muscle injury within rats.[1]

In an interview with *The Scientist*, Sadtler discussed why and how she developed her spectral flow cytometry panel for rats, as well the advantages and disadvantages of spectral flow cytometry.

Q. What is flow cytometry and spectral flow cytometry?

Flow cytometry measures the expression of different proteins on cells one at a time while they are flowing through a machine. You can think of it as marbles through a garden hose. As each marble passes through, there are detectors to say, "this marble has orange in it" or "this marble has purple in it." Those different colors represent the fluorophores' peak emissions and this tells us what proteins are present on the cells.

Everything in life and science is messy. Some fluorophores emit over a variety of wavelengths, so some marbles have red-orange inside them as opposed to just orange or red. Spectral flow cytometry is a take on flow cytometry, which instead of looking at a peak in a specific channel (i.e., the orange channel or the red channel), it looks at the pattern of the whole spectra. This technique allows researchers to tease apart signals from very similarly colored fluorophores.

Q. Why did you generate a spectral flow cytometry panel for rats?

We work at the intersection of immunology and bioengineering, where each field has a different model system. Mice have been the workhorse for immunology for a very long time and as a result there is an absolute plethora of reagents. However, researchers use rats more frequently when evaluating medical devices and biomaterials, but reagent availability is limited. We wanted to bridge the gap between basic, mechanistic immunology studies that happen in mouse models versus the biomaterial studies that happen in rat models. Our goal was to figure out what tools are out there for rats, assemble them into a usable toolbox, and present said toolbox. The spectral flow cytometer allowed us to use similarly colored fluorophores that could not necessarily be used together on a standard flow cytometer.

Q. How did you construct your panel for spectral flow cytometry?

To construct our panel, Kenneth Adusei, the lead author of the paper, and I performed a product search of the various rat antibodies that were available off-the-shelf. We created a reagent spreadsheet that stated which vendors had which antibodies and in which colors. As for choosing which fluorophores to use, we needed to evaluate the autofluorescence of our samples to avoid the peak channels where autofluorescence was coming in.

Q. What are the advantages and disadvantages of spectral flow cytometry?

Spectral flow cytometry allows scientists to distinguish between the real signal of a fluorophore versus what signal might be bleeding through from an overlapping fluorophore. This lets researchers measure more colors at a time with confidence. They get more of the information they want, but also noise from autofluorescence. We work with highly autofluorescent samples, and some of this autofluorescence is from the myeloid cells themselves, which shine bright with pretty colors before we stain them. This gives us an extra variable to deal with. But autofluorescence provides a lot of information as well. For example, we can identify eosinophils by their autofluorescence alone, without any markers. This points to the future recognition of different cell populations based off their autofluorescence. Though the extra noise is a pain, that noise has information within it. We just have to read through the signal.

This interview has been condensed and edited for clarity.

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