Practical Afternoon Hands-on Sessions

Designing Solid-Phase Quantitative Imaging Cytometry Experiments

Lead Instructor: William G. Telford, National Cancer Institute, National Institutes of Health, Bethesda, MD

William Telford obtained his Ph.D. in microbiology from the Michigan State University in 1994 and continued his postdoctoral training in immunology at The University of Michigan Medical School. He joined the National Cancer Institute of the National Institutes of Health (NIH) in 1999 and presently is Director of the flow cytometry core laboratory in the NCI Experimental Transplantation and Immunology Branch. Dr. Telford's research interests include laser scanning cytometry and other areas of instrument and assay development.

Abstract

This workshop will explore the basics of Quantitative Imaging Cytometry as applied to a variety of sample types and assays. Topics will include sample preparation (fixation and staining), the choice of dyes (fluorescent, chromatic, organic dyes, Quantum dots, use of viable dyes, multiplexing), the selection of controls, image analysis criteria and methods, event-based contouring vs. random sampling, cross-channel compensation and data presentation (linear versus log histograms, gating) as well as exporting into various flow cytometry analysis applications. These topics will be explored by examining three specific types of assays: 1) event-based immunophenotyping, 2) confluent monolayer analysis and 3) whole organism analysis.

In event-based immunophenotyping, very low numbers of suspension cells will be immunolabeled using multiple antibodies conjugated to traditional fluorochromes, and analyzed on the iCys® Research Imaging Cytometer. This technique is very applicable when cytometric analysis of small numbers of cells (i.e. a few hundred) is required.

Confluent adherent cell monolayers can often be analyzed as conventional event-based samples, but due to their cell density and complex cell shape this type of analysis can be challenging. We will analyze adherent fibroblasts and ovarian cell line cultures expressing the fluorescent proteins GFP, DsRed and mKate. Issues particularly applicable to confluent cells will be covered such as event-based contouring of closely packed and irregularly shaped objects, including multiple segmenting criterion and watershed algorithms and the use of "random sampling" in confluent cell cultures.

Image cytometry can also be used to analyze whole organisms, such as immature *D. rerio* (zebrafish), *C. elegans* (roundworms) and *Drosophila* larvae. We will analyze both GFP-expressing 7-day zebrafish larvae and *C. elegans* to illustrate the problems associated with the analysis of large organisms, both as single objects and in high-throughput formats.

Multi-color Solid-phase (Adherent Specimen) Cell Cycle Analysis

Lead Instructors: James Jacobberger and Tammy Stefan, Case Western Reserve University and Case Comprehensive Cancer Center, Cleveland OH

Since 1985, James Jacobberger has been on the faculty of Case Western Reserve University where he is now Professor of General Medical Sciences (Oncology), and Associate Director for Shared Resources and Director of the Cytometry and Imaging Microscopy Core of the Case Comprehensive Cancer Center. He has specialized in cytometry as a scientific discipline from his doctoral studies at the University of Rochester onwards, currently focusing on cell signaling and cell cycle processes viewed from a systems orientation. Current research is centered in two enterprises—multi-variate cell cycle analysis and modeling, and leukemia cell signaling—with a long-range goal of creating an analytical system, preferably within a clinical pathology or basic cell biology setting, in which the measurements made at the cytometer are interactively interrogated by the investigator and a computer program with mathematical models of cell biochemistry and integrated systems simulations running underneath.

Abstract
Simple and more complex laser scanning cytometry (LSC) assays for cell proliferation in tissue culture will be demonstrated and discussed. We will use automated statistical algorithms to examine the differences between treated and control cell populations, and also demonstrate how each individual point in a complex data analysis can be traced back to its cellular image.

The objective of measuring cell proliferation is usually to obtain an estimate of the population growth rate, or arrest at a cell cycle checkpoint, as a function of some growth-stimulating or -inhibiting treatment. The simplest precise assay for cultures of exponentially growing cells is a count of cell density and measurement of DNA content. This requires a single DNA-binding dye and outputs the fraction of G1 cells per cm^2. Morphometric features such as chromatin condensation identify mitotic and apoptotic cells. More comprehensive cell cycle analysis is possible by incorporating immunofluorescence. Mitotic and apoptotic cells are differentiated with mitotic markers like phospho-S10-histone H3 (pHH3). Addition of p120, Ki67, or PCNA separates cycling from non-cycling cells, which is useful if quiescent cells contaminate the G1 fraction. Multiplexing with markers that are expressed or degraded out of phase with each other during the cell cycle creates additional cell cycle sub-compartments related to cell behavior. For example, a combination of DNA, pHH3, and cyclins A2 and B1 creates an analytical scheme in which the mitotic stages of prophase, prometaphase, metaphase, and late mitosis can be quantified. This is possible because these cyclins are degraded sequentially by APC^{Cdc20} during prometaphase (cyclin A2) and after metaphase (cyclin B1). The sub-compartments thus created define cell biochemical states. LSC provides morphological validation between mitotic state and behavior by viewing nuclear images.

Immunophenotyping of Small Needle Aspirates

Lead Instructor: William Geddie, Princess Margaret Hospital, University of Toronto, Canada

Abstract

In this workshop we will explore cell surface marker immunophenotyping by Laser Scanning Cytometry. Immunophenotyping by Laser Scanning Cytometry was originally developed by Dr. Richard Clatch in 1998. This method uses a special sample carrier called the *Clatch slide*. This method requires a minimal number of cells and allows for the potential re-use of cells. Specimens are derived from fine needle aspirates (FNA), body fluid or tissue or blood.

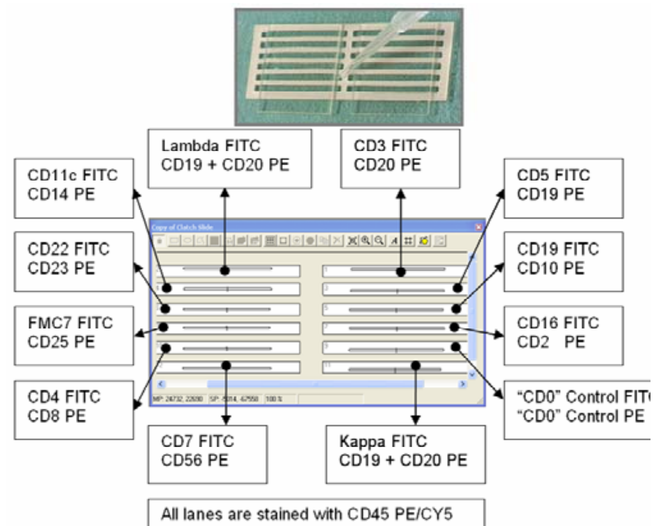
We will cover defining the analysis protocol including appropriate choices of antibodies and fluorochromes, preparation of the sample to satisfy the requirements or constraints of the analytical system, the definition and execution the analysis protocol and the assessment of the quality of the sample preparation and the acquired data.

Participants will perform some of the components of the process using non-biological materials and then will analyze a pre-prepared sample on the iCys.

Advantages of immunophenotyping by laser scanning cytometry that will be discussed are:

- The requirement for a relatively small number of cells,
- A lower cost-per-reportable-result,
- The ease of instituting the method in laboratories that only need to immunophenotype samples on an intermittent basis,
- The use of a simple standardized assay (once set up) that requires few decisions on the part of the technologist, particularly useful for laboratories where there is limited expertise in immunophenotyping,
- Cells are left “in-situ" after data acquisition so that they can be re-analyzed to clarify results or re-stained with other antibodies with or without permeabilization,
- Allows for the recovery of DNA from the analyzed sample (without cell sorting) and permits use of cells for subsequent “FISH" analysis,
- Easily permits cell immunophenotype to be linked to cell morphology.

A typical LSC immunophenotyping panel utilizing the *Clatch slide* is shown below.



Practical Afternoon Hands-on Sessions

Multi-color Quantitative Analysis of Protein / Biomarker Expression in Tissue, Tissue Microarrays and Fine Needle Aspirates

Lead Instructor: Jed Mahoney, Dana Farber Cancer Institute, Boston, MA.

Abstract
The study of protein/biomarker expression in tissue is becoming increasingly key in identifying, understanding and validating the clinical impact of diagnostic-related, prognostic-related, and therapy-related markers. The subjectivity and labor intensiveness of manual scoring methods drives the need for automated methods.

This workshop will teach methods for the automated Laser Scanning Cytometry quantification of biomarkers in chromatically and fluorescently stained tissue. Specifically we will explore 1) clearly defining the desired biological end-points, 2) preparation of the sample, 3) set-up and execution of the analysis protocol, 4) quality control of the sample preparation and the acquired data and 5) outputting and reporting the end-point data.

Sample preparation instruction will include:

- Tissue trimming and fixation,
- Paraffin embedded tissue processing,
- Microtomy,
- Immunohistochemistry and immunofluorescence,
- Specific guidelines for automated (and LSC) analysis.

A “Decision Tree” based analysis strategy will be described that includes:

- Defining the scan type and scan parameters,
- Acquiring and overview scan of the entire sample,
- Acquiring data for the quantification of each marker at high resolution,
- Segmenting the image based on signal levels (event-based and random sampling segmentation),
- Feature extraction – obtaining numerical data from the “segmented events,”
- Population gating to obtain relevant “events,”
- Display of data relationships,
- Numerical data output to Excel,
- Calculation and plotting of end-point data in Excel.