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Photonics Takes on the Challenge of Microanalysis

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The desire to acquire detailed information from specific cells has inspired several novel advances in photonics technologies.

Breakthroughs in photonics technologies have made the field of microgenomics a viable alternative to common genomic studies. Microgenomics may be broadly defined as the molecular analysis of pure cell populations often limited in cell number. The origins of microgenomics grew from the desire of oncologists to determine gene expression patterns from two or more specific sets of cells, such as the genetic expression patterns of oncogenic versus normal cells. Novel photonics technologies such as laser-capture microdissection and microvolume spectrophotometry and fluorometry enable scientists to perform gene expression studies on minute cell populations, or even on a single cell.

The basic microgenomics work flow consists of capturing precise populations of cells, isolating the biomolecule of interest (RNA, DNA or proteins), amplifying and labeling the biomolecules as needed and performing downstream molecular analysis. Prior to the advent of several microvolume technologies, the challenges were daunting: How can specific cells of interest be precisely isolated from a heterogeneous mixture of cells without destroying the biomolecules of interest? Further, if such cells could be acquired, how could the molecular components of interest be isolated in a way that would provide enough material to acquire meaningful data? Finally, how can quality control be performed throughout the process without consuming the precious sample?

Pathologists, as well as other investigators familiar with tissue section examination, can recognize morphologically distinct cellular states under a microscope. Determining the molecular expression profile of these distinct cells is critical to understanding cellular processes, such as oncogenesis.

Separating the cells of interest from a complex tissue matrix that contains cell types in various morphologic states is now possible, thanks to laser-capture microdissection. The technique enables scientists to specifically acquire precise cell populations for genetic analysis (Figure 1). Laser-capture microdissection instruments are available from Arcturus Bioscience Inc. of Mountain View, Calif., and similar technologies are offered by other companies. The Arcturus systems use an infrared laser to adhere selected cells to a thermoplastic film while preserving intracellular biomolecules of interest such as DNA, RNA and proteins. When combined with traditional laser-capture microdissection, an optional UV cutting laser enables the rapid isolation of multiple cells of interest or larger tissue areas.

The volumes used throughout the microgenomic work flow, from laser-capture microdissection to gene expression arrays, are often so small that conventional means of biomolecular quantification are insufficient or unacceptable. Once the de-

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sired cell population has been microdissected from the tissue, quantitation of extracted biomolecules must be performed in a way that consumes minimal sample. Such precise quality control ensures that the desired biomolecules are present at critical steps in the process and helps the investigator determine the optimal course in acquiring sound gene expression data.

By assessing at-strength samples while consuming only 1 µl of a sample, investigators can perform essential quality control checks at various points in the microgenomic work flow. Such critical quality control is not possible with traditional cuvette-based systems. For example, RNA isolated from a limited cell population may be eluted in as little as 10 µl. Most of this material must be subsequently amplified to generate adequate amounts of probe for expression array analysis. Traditionally, investigators would forgo quantitation of the original RNA extract because conventional methods would consume most, if not all, of the sample. However, new microspectrometry and microfluorometry systems such as those from NanoDrop Technologies Inc. of Wilmington, Del., allow measurements of samples as small as 1 µl.

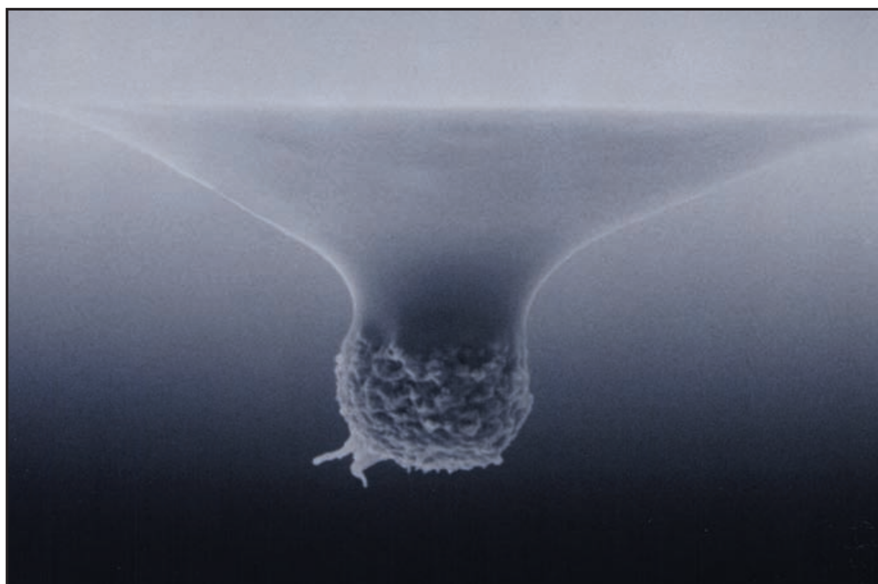

Figure 1. A single cell captured by laser microdissection adheres to a thermoplastic film. Courtesy of Arcturus Bioscience.

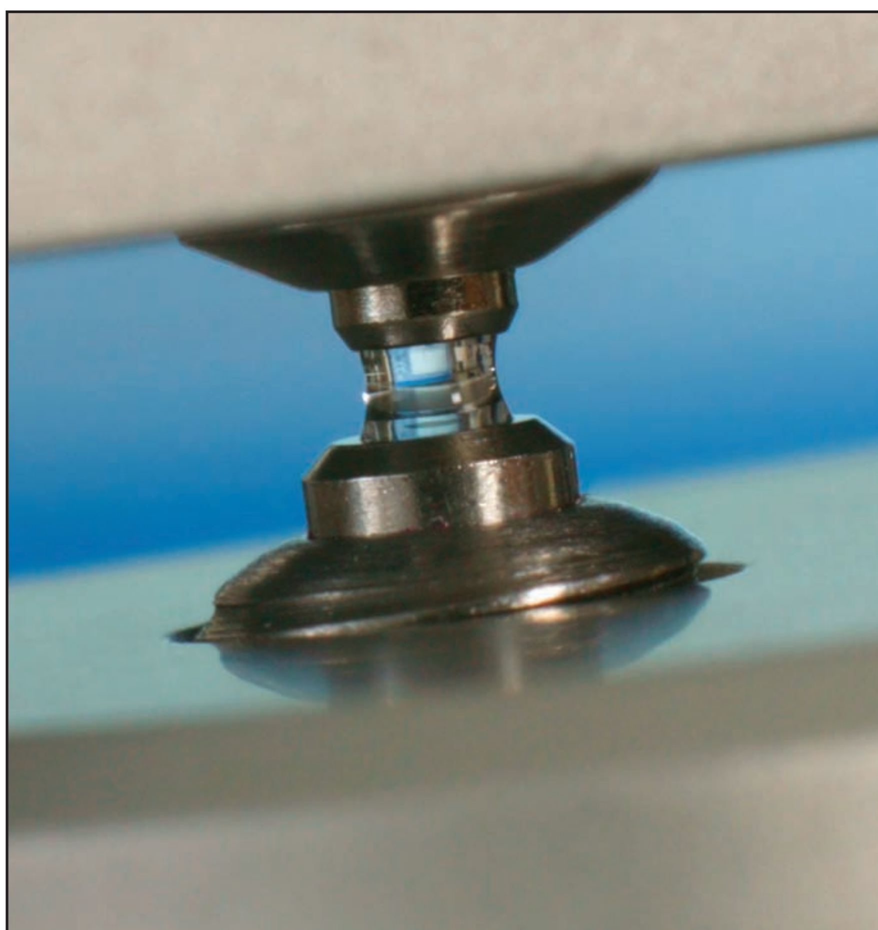

Figure 2. The ND-1000 spectrophotometer uses the inherent surface tension to hold the 1-µl sample between two optical surfaces.

By requiring so little sample, researchers can afford to assess the success of the initial RNA extraction and isolation before committing time and resources to a specific sample that may or may not be suitable. In fact, quality control may be performed at key steps throughout the microgenomics procedure. These include the quantitation of the initial RNA isolation and amount of amplified RNA produced, as well as determining the efficiency of dye incorporation prior to committing a labeled amplified RNA probe to an expression array. This allows researchers to perform microgenomic analysis with confidence.

Samples as small as 1 µl can be analyzed with a system that uses the inherent surface tension of microsamples to hold the sample in place during spectral measurements. This overcomes the barriers posed by conventional containment systems. The first application of the retention system was developed for spectrophotometric measurements.

The system uses a xenon flashlamp for measuring most biological chromophores. The flashlamp's spectrum sufficiently covers the ultraviolet and visible ranges to allow absorbance measurements with adequate sensitivity. It requires essentially no warm-up period, and it fires only during the measurement cycle, greatly expanding the longevity of the lamp. One lamp can be used for ~30,000 measurements (calculated dividing the minimum number of flashes throughout its life span by the average number of flashes required to take an absorbance measurement).

The retention system captures the sample between two optical surfaces by using the inherent surface tension of the sample (Figure 2). The user places as little as

1 µl of the material onto the lower surface and lowers the lever arm of the apparatus. Once the upper surface comes in contact with the sample, a liquid bridge forms between the surfaces, creating a vertical path length.

A solenoid toggles one of the optical surfaces between two mechanically set points, allowing path length adjustment in real time. Once the liquid column of controlled path length is formed, the xenon flashlamp fires ~2000 pulses of light through the sample.

Measurements are taken first at a 1-mm path length and then at a 0.2 mm (Figure 3). According to the Beer-Lambert law, which inversely relates absorbance with path length, shorter lengths will permit higher absorbance measurements. As a result, the addition of the 0.2-mm path allows absorbance measurements 50 times more concentrated than can be obtained with a traditional 1-cm path, cuvette-based system.

Therefore, measuring the absorbance at the two path lengths during the same measurement cycle greatly increases the dynamic range. For example, the 1- and 0.2-mm path lengths used in concert allow concentration measurements of 2 to 3700 ng/µl for double-stranded DNA, virtually eliminating the need to perform dilutions.

Once the light passes through the sample, it is collected by the lower optical surface and sent via optical fiber to an internal linear CCD array. The 2048-element array covers the wavelength range from 190 to 850 nm, but the signal limits the useful range to 220 to 750 nm (about three elements per nanometer). The ability to use only 1 µl of sample combined with real-time path length adjustment provides a powerful way to measure a wide variety of minute samples.

Microfluorometry

Higher sensitivity may be achieved by combining the sample retention technology with fluorescence techniques. The microfluorometry system uses the same retention system to detect emission spectra of common excited fluorophores and can be much more sensitive than direct absorbance measurements. For example, fluorescence emission of PicoGreen dye is enhanced a thousandfold when specifically bound to double-stranded DNA. When compared with direct absorbance measurement of double-stranded DNA, a bound complex of PicoGreen fluorophore and the DNA allows for measurements as low as 5 pg/µl, as opposed to 2 ng/µl using the direct absorbance system.

The system consists of multiple LED excitation sources, the retention system and a fixed CCD detection array. Excitation of bound or unbound fluorophores is generated from three sources: a filtered 365-nm LED, a filtered 470-nm LED and a nonfiltered ~500- to 700-nm (white) LED. The LED sources cover excitation requirements for many common fluorophores used for molecular analysis. The retention system combined with several excitation sources allows versatility for experienced fluorescence users and simplicity for investigators less acquainted with fluorescence techniques.

As with the microspectrophotometry system, a 1-µl sample is placed on the lower optical surface of the retention system and is held in place by surface tension. However, unlike the microspectrophotometry system in which a xenon light source passes through the vertical path of the sample column, LED excitation strikes the column at a 90° angle to its axis (Figures 4, 5). Fluorescence emission is collected via the lower optical surface

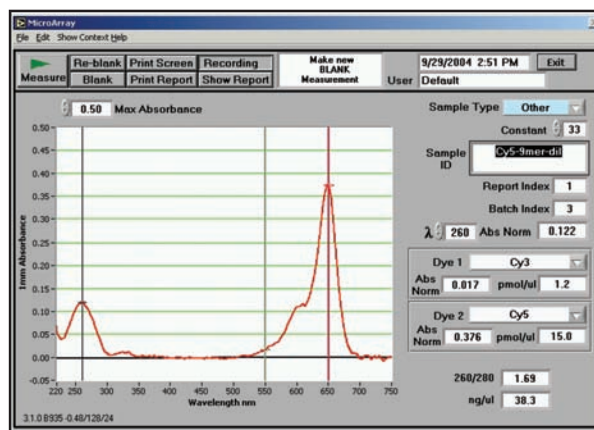

Figure 3. Absorbance measurements taken at two path lengths allow a large dynamic range. Shown here are measurements from the 1-mm path length.

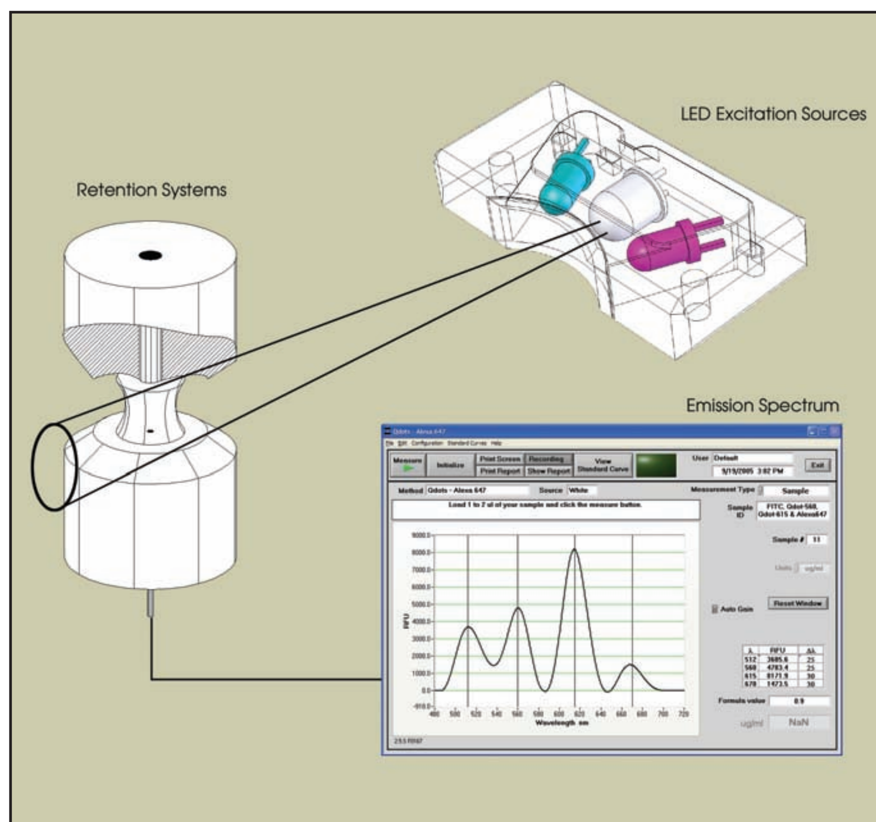

Figure 4. In the microfluorometry system, three LEDs illuminate the sample from the side. The LEDs emit at ~365, ~470 and ~500 to 700 nm (white). The broad emission of the white LED allows several fluorophores to be excited at once, as shown in the example emission spectrum.

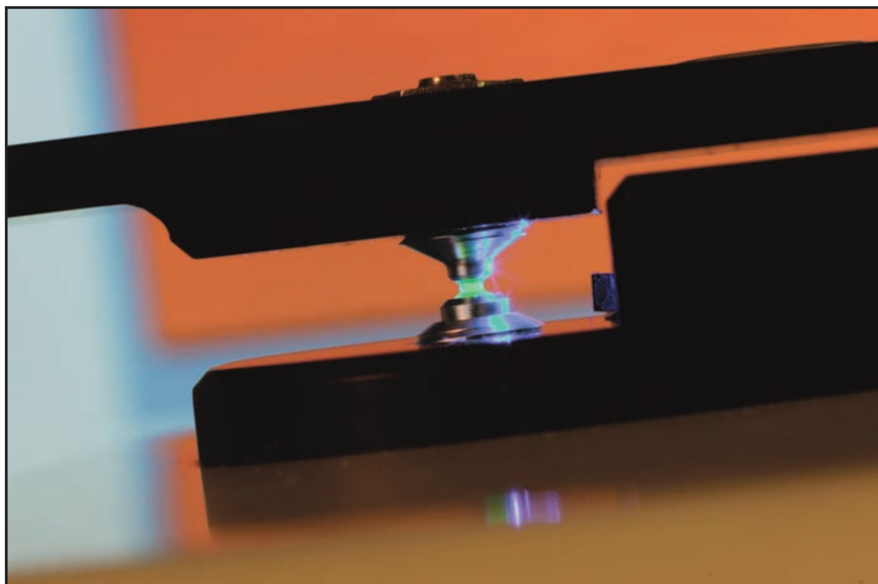

Figure 5. The retention system of the ND-3300 fluorospectrometer holds a sample in place while one of three LED provides fluorescence excitation.

face and travels to an internal CCD array detector, generating emission spectra from 400 to ~800 nm. The white LED, in combination with unique signal-filtering software, provides a large excitation range in which several fluorophores may be assessed simultaneously.

This system can detect several orders of magnitude greater than absorbance spectroscopy, while using minimal sample volume. Fluorescence signal of double-stranded DNA coupled to PicoGreen reagent enables detection at 1 pg/µl. Similarly, for limited cell mass samples whose RNA concentrations are undetectable by absorbance spectroscopy, fluorescence measurement of RNA coupled to RiboGreen reagent lowers the detection limit to ~5 pg/µl.

Applications

The driving force behind microspectrophotometry and microfluorometry is the desire to conserve precious sample while acquiring valuable molecular information, such as concentration and purity. Many biomolecules of interest are chromophores and are commonly assessed using spectrophotometric analysis.

For example, microspectrophotometry can be used for microarray probe development, quantitative reverse transcriptase-polymerase chain reaction (PCR) template normalization, PCR-based microbial identification, and general quantitation of nucleic acids and proteins. Some of the more unique applications include determining the metastable phase for small protein crystallization, extremophile genotyping, forensic analysis, histocompatibilty leukocyte antigen typing for organ transplantation and nanoparticle quality control.

Microfluorometry applications include quantifying even smaller amounts of nucleic acids or proteins using fluorescent dyes, microarray probe quality control, analysis of fluorescent probes such as molecular beacons, sample characterization for microfluidics development and mycotoxin analysis.

New challenges

As methods are moving more toward smaller sample analysis, such as those required for microarrays, the need to perform analysis on large numbers of samples in an efficient manner increases. High-throughput microanalysis is critical in today's research environment. The challenge is to maintain the simplicity of the microvolume systems while adding high-throughput capability in a cost-effective manner.

Just as advances in spectrophotometry and fluorometry addressed the demand of minute volumes, microspectrophotometry and microfluorometry must meet the demand for high-throughput analysis. Designs are being developed to handle multiple-channel measurements, as well as an automated system for a 96-well format.

Scientists have been pushing the limits of current technology to perform molecular analysis on progressively smaller samples. Novel photonics technologies are now enabling researchers to perform gene expression studies on minute cell populations, critical to the understanding of important cellular processes. □

Meet the author

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