Addressing "the three Rs"

Microfluidics can help reduce the number of animals used in research and preclinical studies.

BY CHRISTINA BURTSOFF ASP

In recent years, the advantages anticipated from miniaturization have driven the development of microfluidic technologies, often focused on high-throughput screening. However, screening is not the only step in drug discovery where there's been a dramatic rise in the need to maximize information from small samples and handle complex analytical problems in high numbers.

Preclinical studies requiring small-animal models always present the challenge of performing numerous assays with only a few microliters of sample. Overcoming this problem can involve one or more solutions: limiting the number of assays, diluting or pooling samples to work at the scale or sensitivity of conventional assay formats, extending time courses to monitor biological response over several weeks, and increasing the number of animals used for each experiment. These steps are typically expensive and consume substantial resources.

Furthermore, the last of these solutions is at odds with the animal ethics philosophy known as "the three Rs"—replacement, reduction, and refinement of the use of laboratory animals—followed by pharmaceutical and research establishments.

With these challenges in mind, scientists at the National Veterinary Institute's Department of Immunobiology in Sweden investigated a new approach to protein quantification. The approach uses a compact disc (CD) microlaboratory—namely Gyrolab Bioaffy from Gyros AB (Figure 1, *www.gyros.com*)—to perform sandwich immunoassays using as little as 500 nL for each data point. Preliminary data shows a good correlation between the CD and established macroscale enzyme-linked immunosorbent assay (ELISA) approaches. The CD microlaboratory cut assay times, reduced sample and reagent consumption, and enabled direct detection of cytokines at low concentrations in serum. The results illustrate the potential of a microfluidic solution to significantly reduce the required number of small animals and shorten overall times needed to follow biological responses.

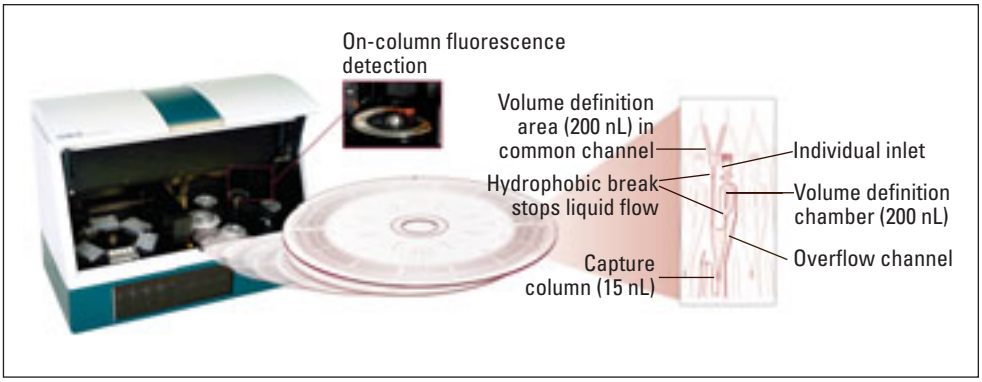

Figure 1. Gyrolab Bioaffy CDs and Gyrolab Workstation LIF. The picture at the right shows the details of a single microstructure. (Courtesy of Gyros AB.)

Vaccine development

Adjuvants are often used in vaccine preparations to induce an inflammatory response and strengthen the immune response. They work, for example, by slowing an antigen's elimination rate to give time for an immune response to begin. An adjuvant often makes it possible to decrease the amount of vaccine required, which is a significant economic advantage. The most commonly used adjuvants stimulate a Type 2 response, one that typically invokes the production of antibodies and cytokines IL-4, 5, 6, 10, and 13. The Type 2 response facilitates the body's fight against bacterial or parasitic infections, but to fight viral infections a Type 1 response invoking the production of T cells, macrophages, and the cytokines IL-2 and IFN-γ is necessary. Achieving a Type 1 response has often proved difficult with current vaccination methods.

But studies at Sweden's National Veterinary Institute found that using ginseng as an adjuvant when vaccinating pigs produces a Type 1 response. The potential for viral protection led the institute's researchers to begin studies in small-animal models.

Existing ELISA formats using conventional equipment with reagents purchased or produced in-house are time-consuming—not only in the duration of each assay but also in the overall time needed to obtain reliable cytokine data. The sample volumes required for each assay severely limit the number of cytokines measurable in a single sample. In addition, since cytokines are very quickly used up by the immune defense system, they are difficult to detect in serum. The researchers found it necessary to wait several weeks, revaccinate the animals, and then measure cytokine levels in cell supernatants of spleen extracts to ensure that a cytokine response had not been missed. To overcome these challenges, they decided to compare the ELISA assays with the CD microlaboratory.

Overall assay times for quantifying 4 different cytokines in duplicate were reduced from 3 days to 4.5 h. Automation of the entire assay, from surface functionalization to final report, decreased the hands-on time from 9 h to 45 min per assay. The researchers also found that working with

integrated processes at the nanoliter scale significantly reduced sample and reagent volumes. For instance, 15 µL of sample was required for the CD assay, compared with 400 µL for ELISA. As shown in Figure 2, the CD microfluidic assay demonstrated a broader measurement range for all three mouse-cytokine standard curves compared with ELISA (although exact comparisons cannot be made because different detection principles were used).

Figure 2 also shows that, with only nanoliter volumes required for each data point, cytokine levels could be measured directly in serum samples. With only 50 µL of serum from each bleed, it was impossible to run duplicate samples and assay several cytokines using ELISA. Samples could not be diluted to overcome the volume limitations because cytokine concentrations would then have dropped below the assay's limit of detection. Preliminary data (not shown) has indicated the presence of elevated IL-2 levels after vaccination, and work is under way to further optimize experimental protocols and confirm the initiation of a Type 1 response with the ginseng adjuvant.

Protein quantification

Each CD used in this experiment contains over 100 individual microstructures with separate inlets and outlets (Figure 1). Each data point is generated within a single microfluidic structure. This eliminates the risk of reagent cross talk and enables a broad diversity of proteins to be quantified within each CD.

A microstructure's specificity is determined by binding a biotinylated capture reagent to a 15-nL streptavidin column. Proteins are captured from crude sample as they pass through the column. Bound proteins are detected by adding a fluorescently labeled detection reagent and then scanning each column. They are quantified by comparison with standard curves produced during the assay. Users can design their own protein panel for single or multiplex assays, using selected capture and detection reagents to define the specificity of each microstructure. For example, one protein can be detected in many samples, or several different proteins can be detected in one sample.

Samples can be assayed simultaneously under uniform conditions, enhancing data reproducibility and reliability. Because sample and reagent volumes are defined in the microstructures, concerns about pipetting precision are eliminated. Liquids move through the microstructures as a result of centrifugal force generated by spinning the CDs at precisely controlled and optimized speeds, ensuring flow rates that maximize capture of specific proteins while minimizing overall reaction time.

The Gyrolab Workstation LIF controls every assay step, from surface functionalization to evaluation. One hundred data points are produced from each CD in less than one hour. A robotic arm transfers reagents and samples from microplates to the CD. As delivery needles are positioned at specified inlets, capillary force draws liquid into the CD microstructures.

To establish the amount of captured protein per column, a laser-induced fluorescence detector scans the columns as the CD spins. Software helps monitor the distribution of fluorescence on each column and calculate protein concentrations. Software visualization of the binding on each column facilitates outlier identification. More importantly, it provides complementary information to the standard curves and speeds up selection of the "best-performing" binding pair during assay development. Assay parameters can be optimized in a minimal number of automated runs so that new assays can be developed within days.

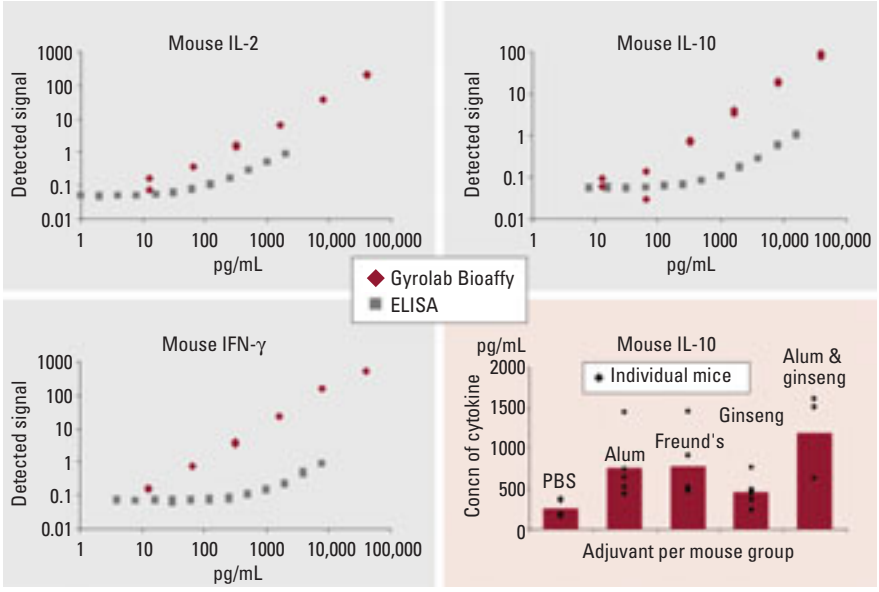

Figure 2. A CD-based assay showed a broader measurement range than ELISA for three different mouse cytokines. The bar chart shows detection of a Type 2 cytokine IL-10 in serum using a CD microlaboratory. Detection by ELISA was not successful because dilution to the required working volumes brought cytokine levels below the limit of detection. (Courtesy of Gyros AB.)

Conclusions

Using microfluidic CD technology to miniaturize and integrate applications allows assays to be performed at scales closer to those existing in biological systems. Working at the nanoliter scale means less sample is required to generate each data point. Scientists pursuing vaccine development have been able to monitor cytokine levels directly in serum samples, which was impossible with their established ELISA platform.

In preclinical studies, using a miniaturized microfluidic solution for protein quantification offers the potential to significantly reduce the number of small animals needed for each experiment and the time required to build up biomarker profiles. Thus, organizations have the chance to save time and money, and to demonstrate support of one of the key principles of the three Rs.

Acknowledgment

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