Using Microfluidics for Metabolic Monitoring of Single Embryo Cultures

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ABSTRACT

In recent years, microfluidics has demonstrated novel methods of culturing and studying cells. Within well-defined volumes comparable to the sample of interest, media, nutrients or toxins may be precisely targeted to individual cells via diffusion or advection, enabled by integrated valves and pumps. Tools, which both enable a repeatable and systematic means of single embryo culture, as well as provide information on metabolic activity of individual embryos, would be of significant utility to the embryologist. To address the need of controlled culture environments and improve embryo health and subsequent positive implantation rates, we have developed programmable microfluidic devices for quantifying metabolite levels in the culture media. Our microfluidic platform provides a high throughput, accurate and repeatable means to non-invasively assess embryo viability, which should be of significant utility to the clinical embryologist.

Keywords: microfluidics, in-vitro fertilization, metabolism

1 INTRODUCTION

It is well established that the developmental potential of preimplantation embryos can be distinguished through evaluation of their metabolism[1-4]. Currently, the most common approach to assess metabolism is to measure changes in concentrations of glucose, pyruvate and lactate in culture media using micropipettes to sample media and perform the assays. This method is technically challenging and labor-intensive. To improve accuracy and throughput of such analyses, we have developed a microfluidic system that can accurately measure concentrations of these three metabolites in nanoliter volumes.

Non-invasive techniques have been pioneered to assess the metabolic activity of pre-implantation embryos during IVF culture[5]. While it is agreed upon that glycolytic rates are correlated with developmental success, metabolic analyses for candidate selection in IVF have not been widely adopted, as existing manual measurement techniques are cumbersome and low throughput. For instance, manual measurements of even ten media samples for a single metabolite using existing microdroplet techniques may take a technician several hours of manned time.

NAD(P)H-based fluorogenic enzymes were used to for automated on-chip assaying of sub-microliter samples of media for select markers correlated with culture viability (glucose, pyruvate and lactate). Microfluidics is particularly suited for manipulation of samples in this volume range and is well poised for automation of the existing protocols. Our metabolite detector platform is scalable to accommodate additional samples and substrates, and most importantly, can be integrated with microfluidic culture systems in the future.

2 EXPERIMENTAL

2.1 Assay Technique

Existing NAD(P)H-linked enzymes assays were used to quantify metabolite concentrations[4, 6]. For every assay it is necessary to combine one part of culture media (typically 1-10nl) with ten parts of a metabolite specific enzyme cocktail. Traditionally, this assay has been performed in microdroplets under mineral oil using custom made glass pipettes. Briefly, droplets containing samples and reagents are dispensed, and allowed to mix by diffusion. Once the reaction has gone to completion (~3 minutes), a fluorescent measurement is manually performed. To increase the throughput of these measurements, an integrated microfluidic device was designed to enable these metering, mixing and measurement operations to be performed automatically.

2.2 Device Architecture

Polydimethylsiloxane (PDMS) devices were fabricated using multi-layer soft lithography which contain channels of micron-scale geometries with integrated pneumatic valves[7] . The chip consisted of a flow layer for manipulating samples, and a complimentary control layer which contained arrangements of valves and pumps. After casting of the two layer PDMS devices, interconnects were punched, devices were cleaned using ethanol and nitrogen, and chips were bonded to glass slides using a plasma treatment. A schematic of the microfluidic architecture is presented in Figure 1. This scalable design accommodates

up to ten media samples, as well as up to six metabolite cocktails and wash buffers.

2.3 Device Operation

The assay system was designed to allow 1nl to be sampled from an input channel and subsequently mixed with 10nl enzyme cocktail. Following mixing, NAD(P)H intensity was measured in a detection chamber through an inverted epi-fluorescent microscope equipped with a DAPI filter set and CCD camera.

Standard calibration curves for each metabolite (glucose, pyruvate, and lactate) were automatically generated on-chip in the 0-1mM range. All operations and data acquisition were enabled by an open source JAVA interface[8]. Parallel systems have been developed in both research and clinical labs.

2.4 Sample Preparation

F1 murine embryos were cultured over four days in individual microdroplets at 5% O_2 using sequential G1/G2 media. On days 1 through 4 of culture, 0.5µl aliquots of media were collected from the culture droplet. Samples were stored in a Petri dish under mineral oil to prevent evaporation. When necessary, dishes containing samples were frozen for at -80°C for later analysis.

Calibration standards of media not containing embryos with known amounts of glucose, lactate and pyruvate in the 0 - 1µM range were also prepared. Standards are loaded on chip in parallel with culture samples to relate observed fluorescent intensity to a metabolite concentration.

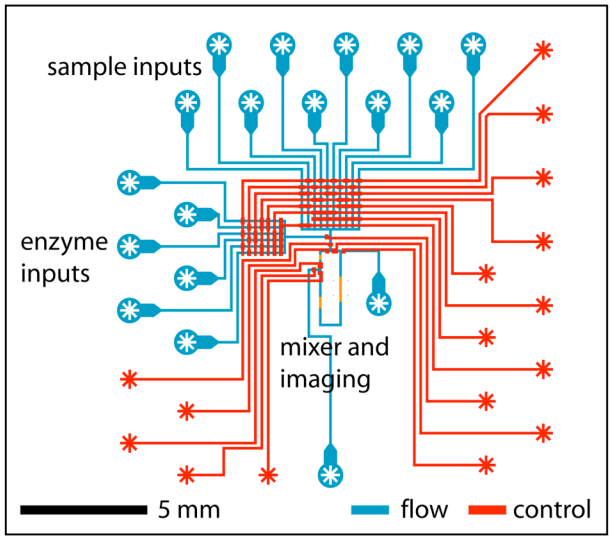

Figure 1: Schematic of the microfluidic metabolite detector. Integrated valves and pumps enable mixtures of samples and enzymes to be automatically generated.

3 RESULTS

3.1 Device Performance

Five-point calibration curves generated for the three metabolites demonstrated linearity from 0.01mM to 1mM (R2>0.999). Figure 2 illustrates an example standard curve obtained in the device using NADH. The microchannels provide a very uniform fluorescent signal and high signal to noise ratio in contrast to the conventional microdroplet assay technique.

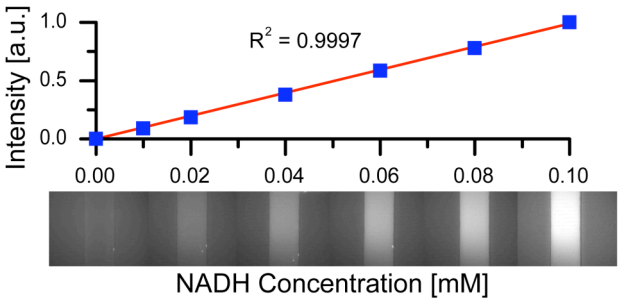

Figure 2: A typical calibration curve overlaid with actual images from the 100um wide microchannels. Similar calibration curves are automatically generated using the on chip mixer for each of the three metabolites of interest.

Each point takes ~1 minute to acquire (including fluid metering and mixing), enabling calibration and parallel sample analysis for three metabolites in approximately ten minutes. In contrast, manual pipetting and measurement generates curves with R2=0.99 at best, has comparable sensitivity and requires at least several fold more operator time.

To contrast the microfluidic approach with the conventional microdroplet technique, several calibration standards with known amounts of glucose, lactate and pyruvate were assayed. Figure 3 compares the results obtained by these two approaches in comparison to the expected concentrations of the metabolite glucose. In general, the microdroplet assays are capable of providing accurate results. However, the microdroplet assays required more than 3h of operator time to obtain results for a single metabolite (a rate of more than five minutes per data point, not including calibration which is required for each enzyme cocktail). In contrast, the microfluidic device performed measurements at a rate of approximately one data point per minute and could process measurements and calibration in parallel. These operations also do not require user intervention.

Figure 4 presents similar measurements performed in parallel using the microfluidic approach with very good repeatability, over ten measurements performed in replicate for each sample and metabolite.

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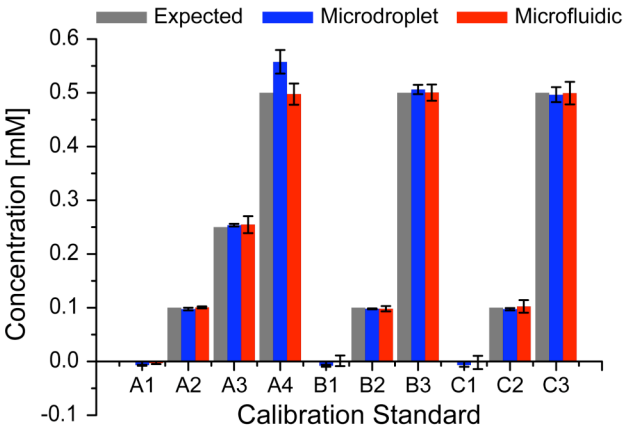

Figure 3: Comparison of glucose measurements obtained using both the conventional microdroplet assay technique and the microfluidic device. Standards A1 through A4 are water based, while standards B and C are based in embryo culture media. Error bars represent one standard deviation on three measurements for the microdroplet approach, and ten measurements on the microfluidic chip.

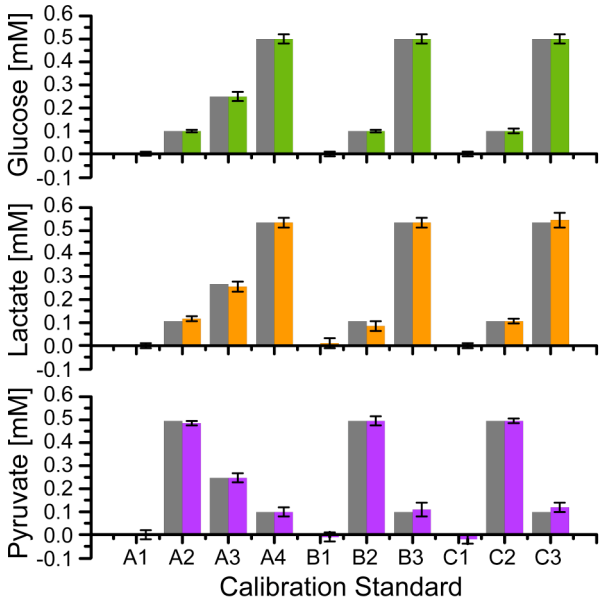

Figure 4: Example results with three metabolites measured in parallel for ten samples demonstrate excellent agreement between expected and measured values. Expected values are indicated in grey and sample names correspond with those in Figure 3. Error bars represent one standard deviation on ten replicate measurements.

3.2 Analysis of Culture Samples

To overcome difficulties in transferring individual 0.5ul media samples from under oil to the microfluidic chip, a dilution approach was employed. Metabolite concentrations of media samples were expected to lie in the 0-1mM range, while calibration studies ensured linearity of measurements of NAD(P)H by-products the 0-10μM range. Thus it was possible to dilute the sample and calibration standards by several orders to increase the working volume.

Media samples were first diluted up to 5μl with water, allowed to mix by diffusion, and then directly collected with a pipette tip. It was possible to inject the sample directly from pipette tips into microbore Tygon tubing, which was then connected to the device input wells. This loading scheme also allowed additional measurements to be performed in replicate sets for each metabolite while avoiding the problems of running out of sample. Calibration studies of different loading schemes enabled robust assays on chip while consuming less than 20nl per measurement.

The error on metabolite assays was also found to be less than 10μM in the 0-1mM range in analysis of multiple murine culture samples. These results suggest that further decreases in analysis run time are possible with reductions in the number of replicate measurements. In conventional assays, measurements are usually performed only in triplicate due to the laborious nature of the work.

4 CONCLUSIONS

A microfluidic device has been developed that greatly improves the accuracy and throughput of metabolite analyses from nanoliter-sized samples. At present, this system can be used as a standalone device for semi-automated determinations of glucose, pyruvate and lactate concentrations in culture media. Work has begun to integrate this device with an on-chip culture system to automate sampling.

The development of a microfluidics-based culture system would undoubtedly revolutionize assisted reproduction. Instead of relying on personnel to monitor the development and transfer embryos to different media, this can be done by using automated, closed chip system. Such high throughput tools will also allow further optimization of embryo culture conditions, and can provide a means to study in depth the correlation between metabolism and embryo viability. We anticipate that an integrated microfluidics approach would be superior to the currently available culture methods. This platform is undergoing continued design and development in research and clinical settings.

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