Published in final edited form as:

Anal Chem. 2008 September 1; 80(17): 6500–6507. doi:10.1021/ac8010473.

Noninvasive Metabolic Profiling using Microfluidics for Analysis of Single Preimplantation Embryos

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Abstract

Noninvasive analysis of metabolism at the single cell level will have many applications in evaluating cellular physiology. One clinically relevant application would be to determine the metabolic activities of embryos produced through assisted reproduction. There is increasing evidence that embryos with greater developmental capacity have distinct metabolic profiles. One of the standard techniques for evaluating embryonic metabolism has been to evaluate consumption and production of several key energetic substrates (glucose, pyruvate and lactate) using microfluorometric enzymatic assays. These assays are performed manually using constriction pipettes, which greatly limits the utility of this system. Through multilayer soft-lithography, we have designed a microfluidic device that can perform these assays in an automated fashion. Following manual loading of samples and enzyme cocktail reagents, this system performs sample and enzyme cocktail aliquotting, mixing of reagents, data acquisition and data analysis without operator intervention. Optimization of design and operating regimens has resulted in the ability to perform serial measurements of glucose, pyruvate and lactate in triplicate with sub-microliter sample volumes within 5 minutes. The current architecture allows for automated analysis of 10 samples and intermittent calibration over a three hour period. Standard curves generated for each metabolite have correlation coefficients that routinely exceed 0.99. Using a standard epifluorescent microscope and CCD camera, linearity is obtained with metabolite concentrations in the low micromolar range (low femtomoles of total analyte). This system is inherently flexible, being easily adapted for any NAD(P)H-based assay and scaled up in terms of sample ports. Open source JAVA-based software allows for simple alterations in routine algorithms. Furthermore, this device can be used as a standalone device in which media samples are loaded or be integrated into microfluidic culture systems for in line, real time metabolic evaluation. With the improved throughput and flexibility of this system, many barriers to evaluating metabolism of embryos and single cells are eliminated. As a proof of principle, metabolic activities of single murine embryos were evaluated using this device.

Introduction

Metabolomics represents an important approach to studying functional biological systems, and provides valuable complementary information to that obtained from gene and protein

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Supporting Information Available

Additional information is available as noted in the text. This material is available free of charge via the internet at <http://pubs.acs.org>.

expression studies. The development of new tools and techniques for the analysis of cellular metabolites (specifically various nutrients and waste molecules generated by living cells) has found important application in exploring directed evolution, drug toxicity and cancer^{1, 2}. Metabolomic studies of single cells are advantageous because the complexities associated with cellular heterogeneity can be avoided. For studies of organisms composed of a single or small number of cells, such as embryos during early development, single cell sensitivity is an absolute necessity.

There is also a strong impetus from the clinical field of assisted reproduction to develop noninvasive methods for evaluating the health and developmental potential of embryos. The current prevailing method for selecting embryos in clinical *in vitro* fertilization (IVF) programs is based on physical characteristics identified through light microscopy. It is well recognized within the clinical community that morphology is not only subjective but a relatively poor indicator of developmental potential³. As a result, more than one embryo is often transferred to the uterus in an IVF cycle, frequently leading to multifetal pregnancies which increase risks to the pregnancy, the mother and the child⁴. In an effort to move toward single embryo transfer, a variety of noninvasive approaches to assess embryonic developmental potential are being developed⁵.

One of the most intensely investigated biologic processes in early embryonic development is metabolism. Studies over the last four decades have revealed that the early embryo undergoes dramatic changes in its metabolism, switching from a low to a high basal metabolic rate⁶. Commensurate with this increase in metabolic activity is a switch in utilization of nutrients, switching from a pyruvate to a glucose based metabolism⁶. These metabolic changes are similar to those seen when a variety of cell types undergo cancerous transformation⁷. Furthermore, it has been demonstrated that situations in which embryonic development is abnormal are associated with alterations in metabolism. Studies of murine embryos have shown that those blastocysts with an elevated glycolytic rate have impaired developmental potential⁸. Energy metabolism may also serve as a biomarker for the developmental potential of human embryos, but no appropriate prospective trials have been performed to date⁹. Much of the reason for the limited data on embryonic metabolism resides in the challenges of measuring metabolites in the small volumes of media that are used for culturing individual embryos.

The most common means of noninvasively assessing metabolism of embryos has been to evaluate what embryos consume and produce through analysis of culture media. Several studies have used fluorometric enzymatic assays to evaluate utilization of the energetic substrates, specifically glucose, pyruvate and lactate. For these assays, the metabolite of interest is a substrate for an enzymatic reaction that either consumes or produces NAD(P)H, which fluoresce when in the reduced form. A number of important metabolites, including glucose, pyruvate, lactate, citrate, and several amino acids can be readily assayed using NAD(P)H enzymatic assays¹⁰. For example, pyruvate is assayed by measuring the consumption of NADH in the lactate dehydrogenase-catalyzed reaction. Due to the very small samples, often in the sub-microliter range, these assays are performed using constriction pipettes that can be calibrated to the low nanoliter range^{11, 12}. The drawbacks of this technology are that the method is incredibly labor intensive and pipette construction is complex. Other recently developed approaches with promise for noninvasively evaluating embryonic metabolism include the evaluation of oxygen consumption using a variety of specialized oxygen probes, amino acid turnover using high-performance liquid chromatography (HPLC), and metabolic profiling using various forms of spectroscopy^{13–16}. All of these studies seem to support the hypothesis that embryos with differing developmental potential can be segregated by metabolic activity, yet much work remains to

be done to determine what specific markers should be evaluated and how they should be detected.

In recent years, an array of alternative microfabricated approaches have been developed also with the shared goal of studying cellular metabolism, many of which are technically more complex and may not be suitable for routine clinical use. Electrochemical microphysiometers have been used for monitoring changes glucose and lactate concentrations in cell cultures using both continuous¹⁷ and discrete¹⁸ fluid flow approaches that necessitate larger sample volumes and are prone to difficult calibration. Scanning electrochemical microscopy (SECM) has been demonstrated as a noninvasive means for studying the metabolism of single cells, though probe fouling, instrumentation as well as calibration can also be challenging. Through SECM, oxygen consumption of single bovine embryos has been measured with relation to embryo viability¹⁹, and custom probes for glucose and lactate have recently been used to generate two dimensional concentration maps surrounding individual cancer cells²⁰. Picoliter chambers with integrated electrodes have been used for the quantification of glucose and lactate changes of stimulated single heart cells^{21, 22}. In addition, devices have also been fabricated for monitoring changes in glucose and lactate from cells attached to beads with NAD-linked enzyme assays²³.

These specialized methods exemplify the challenges involved in developing flexible systems that are compatible and can be integrated with embryo culture. Continued advancement in this field also conveys the desire and excitement and studying metabolism at a single cell level with a robust system. With the goal of improving the ability to analyze media for substrates of energy metabolism, we have developed an automated microfluidic platform for performing NAD(P)H-dependent enzymatic assays.

Experimental

Device Fabrication

Microfluidic detectors were prepared by the process of multilayer soft lithography using PDMS²⁴ (Sylgard 184, Dow Corning). Devices were fabricated using the bottom-actuated valve configuration suitable to closing deep aspect ratio channels²⁵. A three layer photoresist process was used to fabricate the flow mold on a silicon wafer. Briefly, a 20 μm thick layer of SU8 10 (Microchem) was first used to pattern input sieves near the device interconnects, which prevented debris from entering the channel network. Next, a 50 μm thick layer of positive tone AZ 50 XT (Clariant) was aligned and patterned. After development, the AZ was reflowed by placing the wafer on a hotplate at 120°C to create channels with round cross sections followed by a hard bake overnight at 130°C. Finally, a layer of 50 μm thick SU8 50 was aligned to the existing AZ, and used to add new channels segments with rectangular cross sections in the mixing chamber for fluorometric imaging. Sufficiently hard baking the base AZ layer made the resist impermeable to most developers and allowed subsequent processing with SU8. The control wafer for the detector chip was created using two layers of 25 μm thick SU8 10. The second layer of SU8 was patterned over the base layer where valves were located. This made it unnecessary to neck down the width of the control channels where the lines crossed flow channels and enabled faster valve performance. Molds were treated with silane to facilitate PDMS casting. The flow and control layers of the device were cast and thermally bonded at 80°C using 5:1 and 20:1 PDMS, respectively. After curing of the two layer device for approximately 36 hours, input ports were punched with biopsy tools and chips were cleaned using ethanol and nitrogen. Individual detector chips were bonded to pre-cleaned 1 mm thick glass slides using a 30 second air plasma treatment on target surfaces at low RF power (Expanded Plasma Cleaner, Harrick Plasma, Ithaca, NY).

Enzymatic Assay Preparation and Nanodroplet Analysis—Metabolite specific enzyme cocktails were prepared following Leese and Barton²⁶ and Gardner and Leese²⁷. Unless otherwise noted, all reagents were purchased through Sigma-Aldrich (St. Louis, MO). **Glucose Cocktail:** 42 mM EPPs buffer, pH 8.0; 42 μ M DTT, 3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.42 mM ATP and 1.2 mM NADP and 14 U/ml hexokinase/7 U/ml glucose-6-phosphate dehydrogenase (Roche Applied Science, 30 ml/10 ml, Indianapolis, IN). **Pyruvate Cocktail:** 63 mM EPPs buffer, pH 8.0; 0.1 mM NADH; and 75 U/ml L-lactate dehydrogenase (Roche Applied Science, Indianapolis, IN). **Lactate Cocktail:** 0.45 M glycine/0.73 M hydrazine buffer; 4.5 mM NAD and 69 U/ml L-lactate dehydrogenase (Roche Applied Science, Indianapolis, IN). As a comparison, microfluorometric assays were performed using a doubly constricted micropipette as described by Gardner¹². In short, a micropipette delivering a defined volume in the 20–30 nl range was used to dispense the appropriate enzyme cocktail on a siliconized slide overlaid with mineral oil. After taking an initial fluorescence reading using an inverted microscope with epifluorescence and a photomultiplier-based measuring system, a micropipette delivering 1/10 the volume of the cocktail drop (2–3 nl) was used to draw up a sample of spent culture media and add the sample to the cocktail drop. Following a three minute incubation at room temperature, the change in fluorescence was determined. The changes in fluorescence were converted to changes in concentration based on standard curves performed the same day with the known concentrations of substrates, using the same cocktail and pipettes. The pipettes were rinsed in acetone between each sample to prevent cross-contamination.

Embryo Culture and Sample Preparation—Female F1 hybrid (C57Bl/6 X CBA) mice at 5 weeks' age were superovulated with 5 I.U. pregnant mare's serum followed 48 hours later with 5 I.U. of human chorionic gonadotropin (Sigma) and then immediately mated with F1 hybrid males. Zygotes were collected from the oviducts 21 hours after mating and cultured individually in 1 μ l microdroplets of embryo culture media blanketed by paraffin oil in a Petri Dish. Every 24 hours, the embryos were transferred to microdrops of fresh media and the dishes containing the spent media were frozen at -80°C until assays were performed. In this noninvasive approach, sampling is not performed from droplets containing embryos; rather pools of spent media are used for assays. Embryos were cultured in modified G1 medium for the first two days and modified G2 medium for the second two days²⁸. To facilitate metabolic analysis, the glucose and pyruvate concentrations were adjusted to 0.3 mM and lactate was omitted from the media. Media were supplemented with 5 mg/ml human serum albumin prior to culture. All cultures were performed in an incubator at 37°C with 5% O_2 and 6% CO_2 . Developmental progress of each embryo was assessed daily by examining the morphology. Half of each spent media drop (500 nl) was transferred to a replicate plate and then both sets of plates were frozen at -80°C until used for metabolic analysis.

Hardware and Automation

Parallel microfluidic workstations were developed both at M.I.T. and Fertility Laboratories of Colorado. Open source JAVA based software platforms at both locations were used to orchestrate all device operations, image acquisition and data analysis²⁹. **Device control:** Microsolenoids were used to control the integrated multilayer soft-lithography valves, and were controlled by JAVA software as previously described^{29, 30}. Typical operating pressures were 15 psig for the control valves, and 3 psig on sample and reagent inputs. Fluids were interfaced to the device using Tygon tubing (500 μ m ID, Cole-Parmer) and sterilized stainless steel pins (New England Small Tube Corp). **Microscope:** The device was mounted on an Olympus IX-71 epifluorescent microscope equipped with a long working distance 20X objective (NA = 0.45), a neutral density filter (ND 0.25), a Sutter Smart Shutter and a Prior motorized stage. Fluorescent reaction products were imaged through a

DAPI (340 nm/420 nm, EX/EM) filter set. The objective was focused on a microchannel with a rectangular cross section within the mixing section of the chip for image acquisition.

Imaging and Analysis: A cooled CCD camera (Apogee Alta U2000) was externally triggered using JAVA and custom scripts through MicroCCD (Diffraction Limited). 16-bit grayscale images were captured for every sample measurement using an exposure time of 0.5 seconds at 2 x 2 binning and a CCD temperature of 0°C. The shutter was synchronized with image acquisition to prevent photobleaching of reaction products prior to measurements. ImageJ (NIH) routines were integrated into the JAVA control software to analyze acquired images in real time. Area averaged background intensity on the chip was subtracted from fluorescence intensity in the microchannel for each measurement.

Calibration: Calibration standards with known amounts of glucose, lactate and pyruvate diluted in G1 or G2 culture media were used to generate calibration curves, to relate measured fluorescent intensity to metabolite concentration. It was possible to use standards containing known amounts of all three metabolites in the same stock, which reduced the number of required fluid inputs to the device. Metabolite specificity of enzyme cocktails was ensured through calibration studies. Calibration curves were highly linear in the range of 0–1 mM for the metabolites glucose, lactate and pyruvate, consistent with the values expected physiologically in embryo culture media. Detection limits were estimated to be below 10 μ M for the three metabolites of interest using the current imaging settings, although it is straightforward to use a variety of methods such as longer camera exposures or deeper detection channels to provide more signal, if it becomes necessary to extend fluorometric limits in future work. Linear regression values on calibration curves often exceeded $R^2 = 0.999$ (in conventional microdroplet assays, $R^2 > 0.99$ on five point calibration curves are considered sufficient). Standards were interspersed alongside media samples on chip during analysis routines for fluorometric calibration. Slope and intercept values for fluorometric calibration of each metabolite were updated continuously over the course of routines to account for any changes in background light levels, or degradation of enzyme.

Results and Discussion

The microfluidic detector device consists of a flow channel network for loading and mixing inputs, as well as a control channel layer for orchestrating the movement of samples and reagents. Input reservoirs to the chip contain all of the enzyme cocktails, rinse buffer, and ports for loading multiple media samples for analysis. A schematic of the microfluidic detector is shown in Figure 1.

Device Performance

Initial studies were performed with calibration standards to establish the fluorometric sensitivity of the microfluidic detector, which relies on the detection of UV-excited pyridine nucleotides, NADH and NADPH, in enzyme coupled reactions. Inlet and outlet channels on the rotary mixer were arranged to prepare ~1:10 compositions of media samples to enzyme cocktails, and samples were mixed using a rotary peristaltic scheme³¹. To facilitate uniform fluorescence signals, detection channels of rectangular cross section were included in line with the rounded channels containing valves.

Figure 2 demonstrates the high degree of linearity obtained when sampling dilute NADH standards in 50 μ m tall channels. The results of these fluorometric assays with dilute standards, ensures that the optical depth of the channels is sufficient. The field of view imaged covers a channel volume of approximately 2.2 nl. While taller channels were previously tested, 50 μ m tall channels provided an acceptable balance between sample volume consumed and reliable fluorescent signal-to-noise ratio. Rather than performing

measurements in inconvenient oil covered nanoliter droplets, reactions are now completed serially in a fixed channel network.

The amount of embryo media per sample available for assays is extremely limited, driving the need to consume minimal sample per assay to enable replicate measurements. In light of these considerations, a peristaltic pumping scheme regulated the amount of media consumed when transporting media samples from the input wells into the mixing ring³². Sample flowrates were calibrated at a variety of pumping duty cycles (over 10 minute averages) to determine the minimum achievable loading time. Input flowrates were approximately ~0.5 $\mu\text{l/s}$ without active regulation, which reinforces the necessity of this metering approach. While the flow of samples was regulated, regular pressure driven flow was used on reagents and wash buffers to minimize loading time. The on-chip metering sequence and measured flowrates are illustrated in Figure 3, and a movie demonstrating device operations is included as supplementary information.

Every measurement requires metering a volume of media sample, and subsequently actively mixing it with an enzyme cocktail. To increase the throughput of the detector chip, optimization studies were also completed for both metering and mixing operations. The results of the throughput studies are presented in Figure 4. Using glucose standards as mock inputs, the linearity of calibration curves was assessed as a function of the amount of sample consumed. It was found that curves were dependent only on the total volume of sample metered, and not on the rate of metering. While the sample segment of the mixing circuit accommodates only 4 nl, it was found necessary to meter more than this volume from the input channels to ensure the greatest level of linearity, due to Taylor dispersion during transport and potential cross contamination in shared input channels. Figure 4(a) illustrates that at least 15 nl of media per assay should be used to ensure complete filling of the 4 nl chamber. To compensate for any device to device variances and provide a safety margin, 20 nl was metered for each assay which takes approximately eight seconds based on the flowrate calibration. A similar optimization study was performed to determine the minimum required mixing time. The rate limiting process of the mixing operation was both a function of mechanical mixing and the rate of the enzyme reaction after fluids have been fully mixed. Maximum linearity was obtained in approximately 20 seconds of mixing for the three duty cycles tested. By contrast, conventional microdroplet assays are completed by diffusive mixing alone, which imposes a delay of at least several minutes per sample prior to imaging³³. In addition to the metering and mixing operations, injection of cocktail and image acquisition each required approximately one second each. The mixing ring was also flushed with water between successive measurements, which resulted in a total operating time of just over 30 seconds per assay. Complete runs consisting of ten replicate measurements of ten samples (including calibration standards) with three metabolites could be completed unattended in less than three hours. For more complex experiments, the architecture may be scaled to accommodate additional enzymes and sample input lines.

Detection Accuracy and Repeatability

It was observed that the fluorescence measurements of repeated samples would decline over several hours after chip setup. The pyruvate assay was most susceptible, presumably due to photobleaching or oxidation of the NADH present in the cocktail. Microfluidic routines, therefore, were written to ensure frequent calibration of all three metabolites, and values for slope and intercept of linear curves were updated continuously. Culture sample measurements were referenced using interspersed standards loaded onto chip, such to minimize inaccuracy due to diminished signal. A replicate run was discarded if the calibration curve did not exceed $R^2 = 0.99$. The high-throughput performance of the system allowed many (typically ten or more) replicate measurements to be performed for each sample and metabolite to ensure consistency. The ability to calibrate continuously and

perform unattended replicate measurements on samples represents distinct advantages of the microfluidic approach in contrast to the manual droplet technique.

Parallel analysis of mock samples was completed with standards containing known concentrations of glucose, lactate and pyruvate to assess accuracy and repeatability of fluorometric measurements. The data presented in Figure 5 shows very good agreement between nominal and measured values, with accuracy on ten replicate measurements typically within 5%, and standard deviations of approximately 20 μ M across these three metabolites. In addition, calibration measurements were carried out using the same device for several consecutive days (Figure S-1, Supporting Information) to evaluate service life of the device. No significant variation in accuracy or repeatability was observed over several days of operations, provided the device was cleaned and dried after use. This further reduces setup time for a metabolic profiling of cells in a lab setting, since control lines do not have to be reconnected to a fresh device for every experiment. Detectors were typically replaced every couple of days depending on whether any debris was accidentally introduced to the device.

Microfluidic experiments were also performed relative to manual microdroplet assays to enable direct comparisons between approaches. Figure 6 presents the results of glucose assays completed on ten water and media based samples using both a micropipette and the detector. As evident by the comparison to expected values, both methods produced accurate measurements. With reagents and the enzyme cocktail already prepared, however, microdroplet measurements require approximately three hours of continuously manned time to perform a calibration curve and measure ten samples in triplicate. By contrast, the microfluidic device generated measurements at a rate of ~30 seconds per point, unattended. In routine clinical settings involving multiple metabolites and multiple samples, these differences in labor compound quickly. Automated image acquisition and analysis also avoids the inconveniences in manual data logging.

Metabolic Analysis of single murine embryos

As a proof of principle of our microfluidic detector, we performed metabolic profiling of ten morphologically similar murine embryos in a parallel manner. It was particularly important to avoid transferring any of the mineral oil (used to blanket and prevent evaporation of the culture samples) into the detector. Difficulties in moving sub-microliter media samples from Petri dishes to the chip were circumvented by employing a sample dilution scheme to increase the working volume of fluid. All 500 nl sample droplets were diluted to 5 μ l with DI water, and allowed to mix by diffusion. Narrow gel loading round pipette tips (~0.5 mm OD) were found to be ideal for transferring fluid from the oil covered samples directly into segments of Tygon tubing that would be connected to the microfluidic device. This technique also prevented introduction of mineral oil into the microfluidic circuit. The loading process is outlined in Figure 7. Total time required to load 10 samples into the device was approximately 10 minutes. Calibration standards and reference media samples which did not contain embryos were also treated in a similar manner to ensure consistency in the loading and measurement process. Prior detection trials at a variety of metabolite dilutions beyond the expected physiological range were completed to ensure reliable sensitivity when employing this loading scheme.

Individual aliquots of spent culture media that had contained single embryos for 24 hours, as well as reference media that did not contain embryos, were both analyzed to ascertain the difference in concentrations of glucose, lactate and pyruvate. In all cases, measurements were noninvasive since assays were performed only spent media aliquots which had contained a single embryo for a limited amount of time. Embryos are transferred to fresh media droplets daily prior to media collection, which maintains sample integrity and

viability. Media samples from day four were selected for analysis as the embryos are particularly active during this preimplantation period. Each measurement was performed ten times, with calibrations completed automatically for each replicate in approximately three hours. The heterogeneity in nutrient utilization amongst morphologically similar day four embryos is apparent as presented in Figure 8. Overall, our measured values are in good agreement with reported literature values³³. Expected metabolism of glucose, lactate and pyruvate are in the range of -5 , $+5$ and -1 pmol/embryo/hr^{26, 33}, (+ and – denote production and consumption, respectively). Experimental measured values ($\pm$ s.d.) of nutrient utilization for this embryo population are -4.82 ± 1.40 , $+5.70 \pm 2.13$ and -0.92 ± 0.76 respectively.

These data serve to support the utility of a microfluidic detector in a research or clinical setting. Since this assay approach is noninvasive, the integrity of delicate cells is never compromised. It is not unreasonable to consider evaluating hundreds of samples with only a few devices. This would be well within the range required for a large experimental or clinical study. It is also possible to include additional enzymatic assays. A large number of molecules can be detected using three or fewer enzymatic reactions. Other substrates of particular interest that could be measured include asparagine, glutamine and alanine, several of the amino acids that have been shown to identify human or murine embryos with better developmental potential^{13, 14}. With a few modifications, we anticipate that the throughput of the device could be even further enhanced. The number of input ports could be doubled or quadrupled with the addition of just two or four additional control lines added to the multiplexor, respectively³⁴. It would also be possible to design a device with several mixing circuits so that the measurements could be performed in parallel. Such a device would either need to be designed so that the detector bins are within the same field of vision, or a motorized stage would be used to image several distinct locations on the device.

A further step toward automation will occur when this analytic device is placed in line with a culture system as this would eliminate manual handling of spent culture media. Furthermore, it should greatly reduce the volume required for these assays. Several groups have made considerable progress in culturing embryos in microfluidic devices^{35, 36}. One concern that will need to be addressed with using NAD(P)H-based assays is that a high energy excitation illumination is required (wavelengths in the ultraviolet spectrum). It will be necessary to shield embryos from exposure to the ultraviolet light, potentially using carbon black doped PDMS³⁷ or separating microfluidic components³⁸. Alternatively, enzymatic assays that produce products that fluoresce at lower energy excitation could be used³⁹.

While embryologists may adapt to microfluidic approaches in the future as advancements in cell screening^{40, 41} and embryo culture continually evolve^{36, 42–44}, the majority of embryo culture will take place in microdroplet formats for the coming years, particularly in regulated clinical settings. For these reasons a standalone, noninvasive microfluidic metabolite detector is an important development as it may be used in conjunction with standard embryo culture techniques. Our microfluidic approach alleviates the issues involved with electrochemical instrumentation and enables high throughput fluorometric measurements with single embryo sensitivity.

The two principal barriers to widespread adoption of this system are the availability of devices and the need for a fluorescent microscope with imaging capabilities. With the widespread dissemination of lithographic equipment and the growing interest in soft lithography, it is likely that facilities to produce these devices will become more prevalent. Software is currently being developed that will expedite and improve microfluidic device design⁴⁵. Once the molds for a particular device are made, the costs of producing casts are

quite nominal. Many IVF centers have high quality microscopes with imaging systems for micromanipulation of gametes and embryos. In the future, it is quite likely that the device will have integrated illumination and detection capabilities. The remainder of the equipment to run the devices is anticipated to cost less than \$3000, making this technology very competitive in terms of up-front costs as compared to spectroscopic or chromatographic equipment required for other analytic approaches.

Conclusions

The objective of this study was to develop an automated system for noninvasively evaluating metabolism of single embryos. While several techniques have been developed for culturing embryos in microchannel environments, none have addressed the challenge of using these devices to assess embryonic metabolism. By combining the sensitivity and automation enabled by microfluidics with previously characterized enzyme-linked assays, we have developed a practical tool that should have applications to many types of cell culture.

As all microfluidic measurements are automated through software, it is straightforward to record data and create custom assay routines. Optimization of these assays and enzyme cocktails should improve throughput further making such measurements more attractive to other embryologists. With measurement accuracy on par with existing micropipette methods, our microfluidic approach provides repeatability, scalability, and most importantly, the ability to perform assays unattended. Since it is noninvasive, our microfluidic detector is also compatible with any format of cell culture, the mechanics of which can be integrated downstream of other culture devices for closed-loop analysis.

High-throughput tools such as those introduced in this work would be an invaluable asset to understand the specific metabolic factors that play the most important roles during the dynamic embryo culture period, and offer a tractable means to elucidate the relationship with developmental potential. While the three of the most common metabolites have been demonstrated in the present study, noninvasive fluorometric techniques can be expanded to include additional metabolites of interest.

Looking forward, it is desirable to standardize and automate the assay procedure to an integrated culture platform capable of carrying out the metabolic measurements on individual embryos and single cells in parallel for the entire culture period. A better understanding of embryonic metabolism is likely to improve the probability of a healthy pregnancy following single embryo transfer, ultimately leading to reduced numbers of IVF cycles per pregnancy and reduced incidence of multifetal pregnancies. These advances would lessen the financial burden and improve the experience of patients undergoing assisted reproduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are thankful to Bill Thies for significant software contributions. We also appreciate technical assistance from Becky Hamilton in performing the microdroplet assays. Fabrication of microfluidic molds was performed in the Microsystems Technology Labs (MTL) at MIT. JPU was partially supported by the National Science and Engineering Research Council of Canada (NSERC PGS-D Scholarship). MTJ was supported by the National Institute of Health (NIH grant K08-HD047431). DLP was supported by Once Upon a Time (grant to DKG).

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Figure 1. Schematic of the microfluidic metabolite detector fabricated using multilayer soft lithography. Red and blue features denote control and flow layers of PDMS, respectively. Six fluid inputs are reserved for supply of enzyme cocktails and wash buffers, while ten inputs are used for loading media samples and calibration standards. To perform assays, samples of culture media are metered into the central mixing ring and combined with a metabolite specific cocktail. A fluorometric measurement is recorded after complete mixing (camera field of view denoted in purple). Finally, the mixer contents are washed prior to the next assay.

Figure 2.

Example calibration curves and corresponding microchannel images. (a) Assessment of device sensitivity with dilute NADH. (b) Typical metabolite calibration curves and typical microchannel images demonstrates linearity of the microfluidic detector. Scale bars indicate 100 μm .

Figure 3.

Peristaltic metering is used to regulate the amount of valuable sample consumed per assay. (a) Three valves, outlined in blue, are actuated in sequence to meter samples into the mixing ring. The path of a sample through the upper left of the mixer is illustrated with red food dye in frames (i) – (iii). (b) Enzyme cocktail (presented with yellow food dye) is loaded through the opposite path of the ring prior to the mixing operation. Scale bar indicates 500 μm. (c) The flowrate of samples into the mixing ring was calibrated to determine the optimal loading rates and required sample volume. Each data point represents a 10 minute average at an input pressure of 3 psig.

Figure 4.

Optimization of microfluidic metering and mixing operations. (a) Four point glucose calibration curves were prepared under a variety of conditions. The linear R^2 regression of calibration curves is presented as a function of sample volume assayed. Linearity is independent for sample volumes greater than 15 nl, and metering rates follow from Figure 3. (b) Similar linear regressions as a function of rotary mixing with enzyme cocktail prior to fluorometric imaging. Based on these calibrations, 20 nl of sample is metered for every assay, and actively mixed for 20 seconds with cocktail.

Figure 5.

Calibration curves to demonstrate repeatability of ten samples measured in parallel, with sample types A, B and C, based in water, G1 media and G2 media, respectively. Black columns indicate expected concentrations for each metabolite. Error bars represent the standard deviation on ten replicate measurements.

Figure 6.

There is good agreement in accuracy between the conventional microdroplet and present microfluidic analysis for a number of calibration standards with known amounts of glucose. Samples A1 – A4 are water based standards, while standards B and C are based on types G1 and G2 sequential embryo media, respectively. Error bars represent one standard deviation on three measurements for the microdroplet technique and ten measurements for the microfluidic device.

Figure 7.

Sample loading sequence. (a, b) Pre-collected embryo media samples are stored in a Petri dish (35 mm) under oil to prevent evaporation. (c) A gel loading pipette tip is recommended for transfer of individual media samples (typically 1–5 µl working volume) directly into a length of 500 µm ID Tygon tubing. (d) The end of the tubing may be cut to prevent the carryover of any oil prior to adding a sterilized pin. (e) All samples and reagent lines are connected to a detector chip which already is connected to control manifold, then (f) mounted on an inverted microscope for the experiment.

Figure 8.

Metabolic profiles obtained from ten murine embryos, labeled a through j. Results are from day 4 of culture, and are presented relative to original G2 media (analyzed in parallel with culture samples) to ascertain the changes in metabolite levels due to embryo activity. Individual embryos were cultured in 1 μ l of medium G2 for 48h prior to analysis. Heterogeneity in metabolism becomes apparent amongst morphologically similar embryos. Error bars indicate one standard deviation. The mean values for this population are represented by $\bar{x}$, with dashed lines for visualization.