MICROFLUIDIC STRATEGY FOR SPATIOTEMPORALLY RESOLVED MOLECULAR SAMPLING FROM LIVE OVARY SLICES

David S. Dandy, Meghan Mensack, John Wydallis, Charles S. Henry, Chad Eitel, and Stuart Tobet

Colorado State University, Fort Collins, Colorado, USA

ABSTRACT

A prototype microfluidic platform has been developed for spatially and temporally resolved sampling of biomarkers expressed by organotypic tissue slices. The device draws liquid from the sample reservoir through discrete sampling ports into separate analysis microchannels. By monitoring the concentrations of specific biomarkers it is possible to map the concentration profiles of these molecules within the tissue as a function of time. This study uses ovary slices to enable monitoring of key regulators of follicular maturation and ovulation including growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), activin, FGF8, inhibin, follistatin, and betacellulin.

KEYWORDS

Microfluidic sampling, organotypic, spatially resolved, molecular gradients, cell signaling, ovulation.

INTRODUCTION

Infertility is a major problem worldwide for humans with an incidence among women of childbearing age that may be as high as 9%. [1] A major cause of this infertility is likely polycystic ovarian syndrome, or PCOS, [2] that is also noted to have an incidence between 5 and 10%. PCOS is perhaps the most common reproductive endocrine disorder in domestic livestock in addition to women, and may become yet a bigger problem in the future under the influence of a growing number of endocrine disrupting chemicals in the environment. [3,4] Advancing our understanding of ovarian function requires real-time, simultaneous detection of a number of key signaling molecules.

Microfluidics has gained much attention in the world of reproductive biology and assisted reproductive technologies. [5–7] While it has heretofore not been a successful strategy to develop follicles past the pre-antral stage using ovarian tissue 'pieces', [8] our preliminary studies, illustrated in Fig. 1, indicate that the ovarian slice preparation provides a solution to this problem. [9] Ovarian slices provide significant 3D support and microfluidics promises consistent provision of essential nutrients. This may yield an optimal solution for what is highly vascularized *in vivo*, but lacking *in vitro*. Furthermore, within a single ovarian tissue slice, there are follicles at different stages that will respond to specific stimuli, e.g., FSH, in different ways. [10] By virtue of the multi-well sampling capacity of the device used here, our experiments will determine responses to the same stimulus in different follicles in the same slices.

EXPERIMENT

In this study we implement a microfluidic device capable of sampling multiple chemical messengers from the ovary slice. A close up view of a prototype sample reservoir is shown in Fig. 2, whereby an organotypic ovary slice is placed on the bottom surface of that reservoir and immersed in growth media. The sampling ports connect to analysis microchannels (AMs), whose bottom surfaces are patterned [11] with antibodies against specific biomarkers. The AMs lead to a common outlet containing multiple passive pumping reservoirs able to provide long-term, steady-state flows of a variety of complex physiological fluids. [12] The flow rate through the analysis channels is linearly proportional to the number of pumping reservoirs in operation. However, during the long-term experiments (> 24 h) carried out here it is necessary to start and stop flow through the AMs to obtain biomarker concentrations at discrete time points. As illustrated in Fig. 3, capillary forces automatically fill the hydrophilic analysis microchannels when media is introduced to the sample reservoir, and the liquid is confined to those channels by the outlet geometry. Subsequent introduction of buffer (or other simple liquid mixture) to a secondary reservoir results in capillary-driven flow to the pumping reservoir(s), and this flow entrains the liquid in the analysis microchannels, thereby creating steady flow over the sensing regions. The flow will continue until the secondary reservoir is emptied; the duration of flow depends on the initial liquid volume introduced to the secondary reservoir.

Proof of principal experiments have been conducted under continuous flow conditions that demonstrate the ability of the system to accurately map the spatial distribution of multiple analytes within the sample reservoir as a function of time. [13] Figure 4 shows the results from a coupled sampling experiment and micromosaic immunoassay, whereby two distinct proteins are introduced to the sample reservoir and their concentrations measured over time in the 19 AMs. The microchannel concentrations are then used to map the analyte distributions in the sample reservoir.

Flow into the sample ports is complicated by the presence of the tissue slice on reservoir floor. To facilitate uniform flow under the tissue, the slice rests on support posts fabricated into the reservoir. Figure 5 illustrates the predicted flow streamlines passing around and under the tissue slices before leaving through the sample ports. The computational fluid dynamics (CFD) simulations also quantify the effect of support post height on the analyte concentration entering the sample ports. These simulations are being used to guide reservoir design so that optimal flow rates and flow paths are obtained under the flexible tissue slice.



Figure 1. Individual frame video sequences showing live GFP fluorescence in granulosa cells driven by the estrogen receptor beta promoter. Tissues are *in vitro* organotypic slices from prepubertal ovaries placed *in vitro* and treated with FSH.

Figure 2. Close up of the sample reservoir. The ovary slice rests on the bottom of the reservoir, atop the vertical sampling ports.



Figure 3. The spatiotemporal sampling device couples capillary valves with our passive pumping strategy [12] to start, maintain, and stop flow. Steady flow continues until the secondary reservoir is emptied; the duration of flow depends on the initial liquid volume introduced to the secondary reservoir. At the desired time point flow can be restarted in the analysis microchannels (AMs) by pipetting a new liquid volume to the secondary reservoir.



Figure 4. The spatial microfluidic assay was reversibly bonded to a substrate with antigens immobilized in discrete stripes orthogonal to the analysis microchannels. Two separate 0.3 μ L injections of anti-rat and anti-rabbit (0.67 mg mL⁻¹) were injected in different locations into the sample reservoir. Steady state flow through the μ FN was $Q_{ss} = 0.59$ nL s⁻¹. The fluorescent images on the left show good spatial resolution between the two analyte species. (B) The fluorescent image is then quantified, corrected for background, and used in a interpolation scheme to estimate the species spatial distribution in the sample reservoir. Three assays of t = 10, 20, and 30 minutes are shown. [13]



Figure 5. (A) Finite element simulation of an analyte ($D = 10^{-11} \text{ m}^2 \text{ s}^{-1}$, $J = 0.01 \text{ nmol cm}^{-2} \text{ s}^{-1}$) being secreted from a tissue slice sitting on the spatial sampler with 4 sampling ports on posts of height $H = 100 \mu \text{m}$ with each sampling port pumping fluid at a rate of 1 nL/s. Analyte is secreted from both the top and bottom side of the tissue. (B) Concentration of analyte in each port vs. time for different post heights.

A key issue is whether the tissue will remain alive and viable in the sample reservoir for extended periods of operation. This issue is non-trivial, given the potential chemical interactions between the tissue and PDMS structures, and the need to minimize evaporation rates from the sample reservoir while maintaining dissolved oxygen concentrations necessary for physiological function. To investigate this matter a live, 200 µm thick section of ovary is cut from a live mouse using a vibrating microtome (Leica VT1000S), and placed in the sample reservoir. Serum free media is then added to the tissue well and the device is placed in a 5% CO2, 37°C environment to maintain viability. A similar environment is maintained while viewing the tissue on the microscope. Using this and other similar slices with 75 µm diameter sample ports on a 150 µm pitch, measurements have been made to



Figure 6. An ovary slice is placed in a sample reservoir between two sampling ports. The analysis microchannels may be seen to the left and right of these two ports. The ovary is immersed in growth media and kept at 37°C in an incubator for 24 h. The slice was fully functional at that time point.

quantify levels of regulator molecules at different locations in the ovary slice.

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CONTACT

David S. Dandy, +1 970-491-7437 or dandy@colostate.edu.