BRIDGING THE GAP: TOWARDS MICROFLUIDIC SINGLE CELL ANALYSIS OF IN VIVO STIMULATED CELLS

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ABSTRACT
We present a novel strategy to enable cellular analysis of in vivo models in microfluidic devices. The strategy combines microdissection of tissue samples and microfluidics for cell capturing and analysis. The technique is optimized for cell capture and analysis of bone, so spatial information can be linked to important information concerning individual cells’ microenvironments, such as strain energy density. In contrast to state of the art microfluidic devices for cell analysis, which are mostly limited to the use of cell suspensions, the presented study opens up the possibility for cell-directed analysis of in vivo models.

KEYWORDS
Microfluidics, Laser Capture Microdissection, Mechanobiology, Bone Adaptation, Osteocytes

INTRODUCTION
Cell analytical platforms are commonly based on the analysis of cell culture in artificial environments [1-3]. Although these approaches mimic natural cellular environments in vitro [4], it is not possible to recreate all of the complex structural and biochemical interactions that occur in vivo. The complex mechanical interaction between cells and their extracellular environment is especially important in trabecular bone adaptation processes, in which dynamic mechanical loading directs bone formation and resorption responses at the micrometer scale towards an optimal balance between strength and minimal weight. In vivo model analyses of bone adaptation frequently use methods such as micro-computed tomography (µCT), multiscale in-silico modeling, and protein and gene expression analysis by immunohistochemistry (IHC) and bench-top PCR approaches, respectively [5]. Despite numerous outcomes in mechanical systems biology, these methods still lack in quantification (IHC) or specificity (PCR) at the single cell level. Here, we report on an approach aiming to bridge the gap between standard in vivo model analyses and the numerous capabilities microfluidics technologies are able to offer.

CONCEPT
The complete strategy is illustrated in figure 1. A vertebral sample of an in vivo mouse model is harvested and prepared for microdissection. Single osteocytes are cut out from the sample section and catapulted into the microfluidic chip by means of laser capture microdissection (LCM). The microfluidic device is fixed above the sample section with open channel structures facing the sample. After cell transfer into the capture chambers, the chip device is removed, and the channel system is closed. Using standard microfluidics for reagent supply, cellular DNA/RNA can be visualized for the characterization of the samples in terms of cell number and integrity.

As each cell sample is captured in a separate microfluidic chamber, subsequent cellular analyses can be linked to individual osteocyte locations within the in vivo model. Thus, retrieved cellular data allows the reconstruction of the sample, e.g. by intrinsic information on gene expression profiles.

Figure 1: Conceptual schematics. (a) A bone sample (mouse caudal vertebra) is taken from a mechanically stimulated in vivo model. (b) Single osteocytes are dissected out of the bone cryosections by a focused laser beam and catapulted into a microfluidic device placed above the sample. (c) On-chip single-cell analysis provides information on the genome or proteome level for every single cell. (d) Local information of the cells is preserved allowing reconstruction of the sample.
The transfer of cells from the tissue section to the chip device is visualized in figure 2. The cross-sectional view in figure 2a depicts the dissection and catapulting step. Individual cells are optically identified and can be chosen according to µCT derived strain energy density maps. Osteocytes of interest are cut out using a high-intensity, focused laser beam and subsequently catapulted by defocused laser pulses into cell capture chambers of the microchip positioned above. The cell capture chambers are connected by microfluidic channels for fluid supply (Fig. 2b), forming an array of 12 adjacent capture chambers for parallel analysis.

**EXPERIMENTAL**

The master mold for the chip device was prepared using a 4” silicon wafer. The fluidic mold was fabricated according to [6] apart from the following: first, a positive photoresist (AZ-9260, AZ Electronic Materials) was used to generate 12 µm high round interconnection channels. In a second step the 100 µm high cell capture chambers were fabricated applying a negative photoresist (SU-8 2050, MicroChem Corp.). The polymeric chip was made using standard soft lithography methods applying poly(dimethylsiloxane) (Dow Corning) in a 1:10 curing agent to pre-polymer ratio.

The 6th caudal vertebrae of C57BL/6 mice were harvested, snap frozen, and cryosectioned (12 µm thickness) according to [7]. LMD tape (Kawamoto) was used to preserve section morphology and facilitate microdissection. In order to identify osteocytes prior to microdissection and to verify the cell tracking potential of this strategy, cryosections were stained with Hoechst stain (10 µg/ml) to mark the nuclei of embedded osteocytes. A laser capture microdissection instrument (Palm MicroBeam, Zeiss) was used to cut out single or multiple osteocytes and catapult these cells into open microchambers. The PDMS chip was attached to a metal holder above the histology section, and a microchamber was aligned just above the osteocyte of interest prior to microdissection.

After cell dissection, the microchambers were closed by a glass cover slip deploying a custom designed clamp. The chip was connected to tubing and a custom-made pressurization system, allowing for the subsequent supply of reagents and staining fluids. Propidium iodide solution (1 ng/ml) was flushed into the channels to identify the osteocytes and characterize their integrity within the transferred sample. Fluorescent imaging was performed with an Olympus microscope (IX70) and an EMCCD camera (iXon, Andor Technologies).

**RESULTS AND DISCUSSION**

The performance of the microdissection process was characterized. First, the scattering of cells upon vertical catapulting onto the microdevice was determined as illustrated in figure 3a. The histograms in figures 3b and c depict the x and y-axis diversions of 142 samples after catapulting, respectively. Based on these findings, we designed the target microchambers with a diameter of 600 µm. Next, the transfer efficiency of cells from the sample into the microfluidic chambers, i.e. dissection and catapulting, were determined to 48%. In 95% of these cases, we spotted intact, not ruptured cell clusters in the chambers. About 75% of single dissected cell samples in the chambers contained complete transferred cells.

**Figure 3: Characterization of the LCM performance.** Cells that are catapulted from the sample reach the wells at different positions in the x-y plane (a). The histograms in (b) and (c) are representative data from 142 dissection and catapulting processes. The marked range in (b) and (c) depicts the diameter of the chip wells, into which the samples are catapulted.
Figure 4 illustrates the sample dissection and characterization procedure. Cell clusters (e.g. fig. 4a, 7-10) and single cells (e.g. fig. 4a, 1-6) from distinct bone regions were collected out of the histology section. Negative samples were included to characterize the background signal of the supporting tape (e.g. fig. 4a, 11-12). Figure 4b represents a selection of processed on-chip samples verifying intact DNA/RNA information for cell samples after the LCM procedure (fig. 4b, No.9 and No.5) and low background signal intensity (fig. 4b, No.11).

CONCLUSION AND OUTLOOK

Our results demonstrate that we successfully combined LCM and microfluidic technologies to bridge the gap between in vivo models and cell analytical microfluidic platforms. This method can be employed for other tissues, cell cultures, and biofilms. In future studies, we plan to investigate mechanically induced gene expression using qPCR in the microfluidic device to yield spatio-temporal information at single cell resolution.

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