A Multiscale Device for the Study of Compartmentalized Purinergic Signaling[1]

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ABSTRACT

Many cell types communicate by means of dendritic extensions via a multi-tiered set of geometric and chemical cues. Until recently, mimicking the compartmentalized *in vivo* cellular environment of dendrite-expressing cells such as osteocytes and motor neurons in a spatially and temporally controllable manner was limited by the challenges of *in vitro* device fabrication at submicron scales. Utilizing the improved resolution of current fabrication technology, we have designed a multiscale device, the Macro-micro-nano system, or Mμn, composed of two distinct cell-seeding and interrogation compartments separated by a nanochannel array. The array enables dendrite ingrowth, while providing a mechanism for fluidic sequestration and/or temporally-mediated diffusible signaling between cell populations. Modeling of the Mμn system predicted the ability to isolate diffusible signals, namely ATP. Empirical diffusion studies verified computational modeling. In addition, cell viability, dendrite interaction with the nanoarray, and cellular purinergic response to heat shock were experimentally evaluated within the device for both osteocytes and motor neurons. Our results describe a novel *in vitro* system in which dendrite-expressing cell types can be studied within nano-environments that mimic *in vivo* conditions. In particular, the Mμn system enables real-time observation of cell to cell communication between cell populations in distinct, but fluidically coupled regions.

BACKGROUND

- Nanoscale interactions among cells and extracellular signaling molecules are essential elements of numerous biological processes including cell adhesion, migration, proliferation, and ***apoptosis***.
- Cells with such interactions are those that utilize arrays of dendritic cell processes to achieve highly selective pathways of intercellular communication, e.g. cells of the nervous system, dendritic cells within the immune system, and ***osteocytes*** in bone (Fig 1).
- For dendrite expressing cells, the fundamental challenge is to create an environment *in vitro* that allows identification and study of specific cellular interactions mediated by cell processes or dendritic networks.

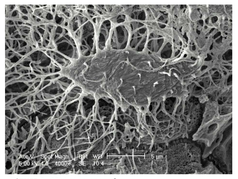

Figure 1. SEM of osteocyte[2]

- Mimicking the system includes controlling geometrically confined cellular spaces and chemical cues such as the diffusion of paracrine signals through a porous medium[3]:

$$\ln\left(\frac{C_0-2C}{C_0}\right) = -\left(\frac{A\cdot D}{V\delta}\right)t$$

METHODS AND MATERIALS

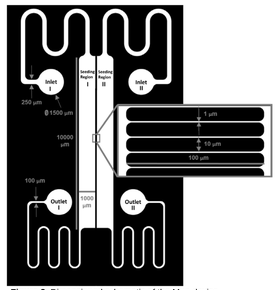

Figure 2. Dimensioned schematic of the Mμn device.

- The Mμn was designed in AutoCAD and fabricated by photolithography on Si wafer using SU-8 negative resists. The mold was silanized to ease mold release, and PDMS was used to create functional devices bonded to glass (Fig 2,3,4).
- The device was modeled using COMSOL for fluid flow profile, heat transport, and diffusion of exogenous small molecules.
- Diffusion was modeled experimentally using fluorescein and rhodamine under static and dynamic flow conditions.
- MLO-Y4, osteocyte-like cells, and B35 motor neurons were seeded within the Mμn on a fibronectin surface coating and assessed for morphology.
- Localized heat shock within the device was used as a method of inducing apoptosis, which was measured by caspase cleavage dye.
- ATP release was measured as a function of unheated vs unheated regions of the Mμn by firefly luciferase bioluminescence assay.

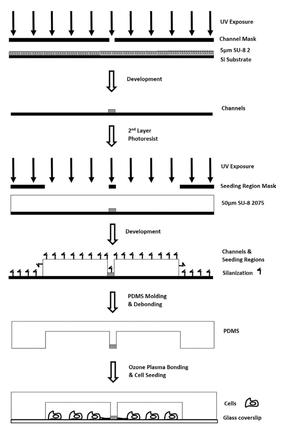

Figure 3: Mμn fabrication process by 2-step soft photolithography.

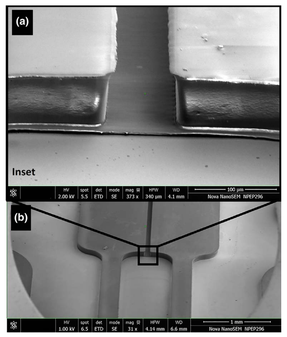

Figure 4. SEM of Mμn illustrating the 2 tier structure in developed SU-8 on Si with inset (a) and wide view (b).

- P2X7 receptor expression was evaluated by immunocytochemistry (ICC).

RESULTS

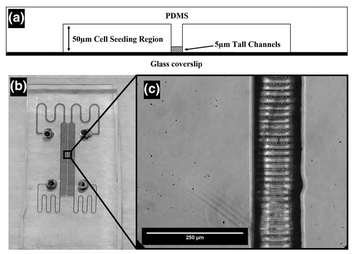

Figure 5. Side view illustration of Mμn device cast in PDMS and bonded to a glass slide (a) with fabricated PDMS device (b) and channel array inset (c).

- The Mμn was successfully fabricated in SU-8 negative photoresist on Si, then molded in PDMS, with measured channel widths of 930nm +/- 350nm (Fig 5).
- The sufficient bonding of the device and fluidic separation of the seeding regions were confirmed by parallel flow of dye within the device.

RESULTS

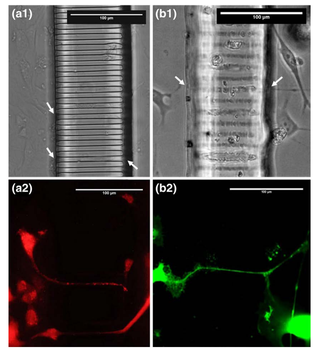

Figure 6. MLO-Y4 osteocyte-like cells imaged in brightfield and with RANKL-reporter construct (a1,a2) and B35 motor neurons imaged in brightfield and with Calcein-AM (b1,b2).

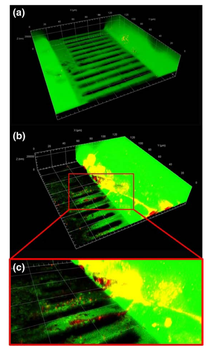

Figure 7. Fluorescein diffusion across the channel array in unseeded device (a) and MLO-Y4 seeded device (b,c).

- Both motor neurons and osteocytes were shown to maintain viability in the Mμn (>90%) and extend their dendritic processes into the channel array (Fig 6) without chemokine induction. Osteocytes at ~60% and neurons at ~25%.
- Diffusion within the device was computationally modeled and experimentally quantified (Fig 8), showing a decrease in small molecule diffusivity with the addition of osteocytes (Fig 8, Tab 1).

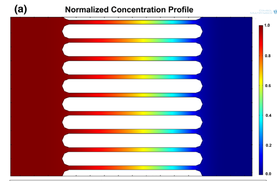

Figure 8. Computational (a) and experimental concentration profile of small molecules in the Mμn with parallel flow (b).

Molecule	MW (Da)	Theoretical diffusivity ($\mu m^2/s$)	Experimental diffusivity ($\mu m^2/s$)
Rhodamine 6G	479	414	255.56 +/- 11.6
Fluorescein	376	425	290.53 +/- 25.0
Rhodamine 6G + MLO-Y4	-	-	191.67 +/- 15.8*
Fluorescein + MLO-Y4	-	-	182.78 +/- 8.1*
ATP	551	302	-

*$p < 0.05$ between respective tracer diffusivities of cell-seeded devices and devices with no cells

Table 1. Theoretical and experimental diffusivity of small molecules in the Mμn.

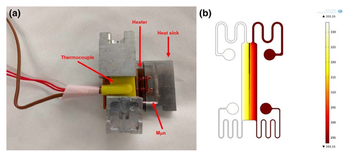

Figure 9. Heating setup (a) and computational heating model at 60° C for 30s (b).

- Heat-stress was used to induce apoptosis in the Mμn using a heating element and heat sink. Computational modeling predicted the isolation of the effect to one side (Fig 9) and was confirmed by caspase 3 measurements.
- Extracellular ATP concentration was shown to increase in the unheated side of the device over 12 hrs for osteocytes undergoing apoptosis, but not motor neurons (Fig 10).

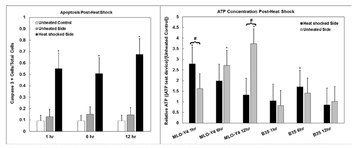

Figure 10. Apoptosis in the Mun at 1, 6, and 12 hrs post heating for all cells (left), and extracellular ATP concentration for each cell type at 1, 6 and 12 hrs (right). *p<0.05 vs unheated control.

- ATP receptor, P2X7, was upregulated on both the heated and unheated side of the Mμn at 12 hrs post heat treatment in MLO-Y4 osteocyte-like cells as visualized and measured by ICC (Fig 11).

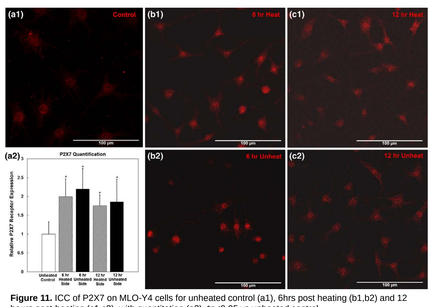

Figure 11. ICC of P2X7 on MLO-Y4 cells for unheated control (a1), 6hrs post heating (b1,b2) and 12 hours post-heating (c1,c2), with quantitation (a2). *p<0.05 vs unheated control.

DISCUSSION

- The Mμn system readily mimics the physical space through which dendritic processes interact *in vivo*, and therefore may promote increased phenotypic similarity and cell function of dendrite-expressing cells.
- Experimental results validate the computational model used for ATP diffusion under parallel flow to within 10%, highlighting the Mμn system's versatility in using parallel flow for control of extracellular signaling molecules and evaluating their impact on cells.
- Experimental free diffusion measurements indicate that diffusion across the array is consistent with the expected values for each molecule. The decreased diffusivity upon cell seeding is expected given the decreased cross-sectional area for diffusion due to process ingrowth and resultant partial channel obstruction.
- Osteocytes demonstrated a higher interaction efficiency than neurons, likely due to phenotypic differences between the cell types. Traversing of the channel array structure by extensions of both cell types indicates the potential for cell to cell interaction between regions, which is readily initiated using the Mμn system.
- Post-heat shock increases in extracellular ATP for osteocytes was paired with elevated P2X7 receptor expression on both sides of the device, showing the Mμn can be used to measure paracrine purinergic signaling across the array.

CONCLUSIONS

- We have designed and fabricated a fluidic device that functions at the macro, micro, and nanoscale to recapitulate the geometric and chemical cues seen by cells with dendrite-mediated communication *in vivo*.
- This device enables evaluation of dendrite-mediated cell signaling, over time, in response to controlled transport of ATP-like molecules.
- Data illustrate that two distinct cell types, osteocytes and motor neurons, remained viable and interactive within the device for up to 7 days. Data also show that release and diffusion of purinergic signals as well as cellular response, can be quantified within our device.
- These results underline the Mμn system as a versatile platform for the study of dendritic cell signaling *in vitro*. In addition, the range of geometric scales will further enable insight into immediate and downstream cell behaviors governed by dendrite-based cell to cell communication, such as the role of osteocytes in osteoclastogenesis.

Future Work

- Examine the connectivity of osteocytes traversing the Mμn by quantifying gap functionality.
- Develop gene reporter systems, specifically RANKL in osteocytes, to quantify the apoptotic response of cells to heat stress, shear stress, and nutrient deficiency.
- Examine the mechanism of ATP bolus release as well as the spatial and temporal resolution of the apoptosis-induced purinergic "find me signal."

REFERENCES AND ACKNOWLEDGEMENTS

References.
1. McCutcheon et al. Biomedical Microdevices.
2. Pajevic. *IBMS BoneKEy*, 2009.
3. Podichetty et al. Biotechnol. Prog. 28, 1045–1054 (2012).

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