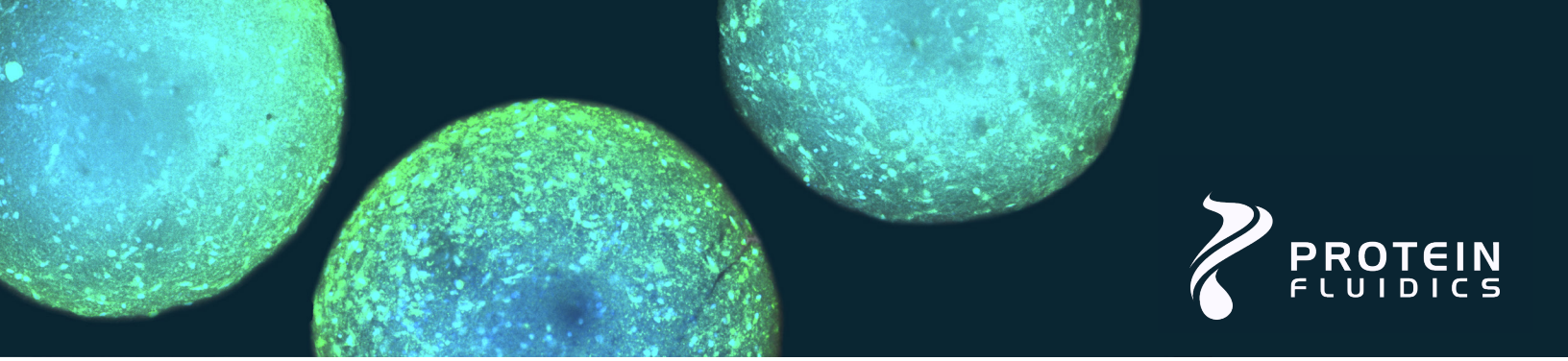

APPLICATION NOTE

Pu·MA System® 3D: Automated Sequential Drug Addition Workflow and Analysis for Neurospheroids

Introduction

3D cell models and organoids provide a better representation of *in vivo* tissue or organ function. For the last three decades researchers have been perfecting the formation and maintenance of various 3D models for understanding both disease and normal physiology (1-3). However, they have been limited by their ability to perform complex assays easily and quickly with these precious samples especially patient-derived material. When manually performing drug treatments and assays in multiwell plates one is often confined to a single readout per sample. For imaging, the manual treatment, staining, and processing of spheroids and organoids is typically labor-intensive and prone to disruption or loss of samples.

The focus of this application note is, the use of our automated Pu·MA System for the sequential drug treatment of neurospheroids for functional evaluation. Neurospheres treated with various neuro-active compounds were analyzed for neuronal activity by calcium oscillations using calcium sensitive dyes and fast kinetic fluorescence imaging. This enabled automated multi-dosing protocols with multiple reagent exchanges for single neurospheroids assay.

Pu·MA System Workflow

Pu·MA System and flowchips have been designed for a streamlined workflow as shown in in Figure 1. which consists of:

- loading spheroids and reagents into the flowchips
- placing the flowchips into the Pu·MA System (inside the incubator)
- running automated reagent exchange protocols via an intuitive touchscreen interface
- analyzing the spheroids in the flowchip with systems such as imagers, kinetic fluorescence systems or plate readers

The system architecture and use of pneumatic microfluidics to move fluids provides gas exchange to the sample chambers within the incubator environment.

The flowchip features include:

- a convenient multi-well plate format (384-well spacings SLAS/ANSI standard)
- standard spacing for use with multichannel pipettes or automated liquid dispense system
- up to 32 tests per plate
- optically clear bottoms for imaging with any fluorescence or confocal imaging system

Figure 1. Schematic of the Pu·MA System workflow for automated organoid assays, *in situ* staining and imaging.

Sequential Drug Treatment

An automated assay protocol for sequential drug addition and analysis of neurospheroid was done using the Pu·MA System (schematic shown in Figure 2). Neurospheroids (microBrain® from Stemonix, Inc.) which contain astrocytes and neurons were stained with Calcium 6 dye and added to the flowchips within the Pu·MA System.

As shown in the workflow the treated neurospheroids in the flowchips were removed from the Pu·MA System intermittently for fast kinetic fluorescence measurements (using FLIPR® Penta High-Throughput Cellular Screening System) and the neurospheroids were imaged within the flowchips using ImageXpress® Micro Confocal Imaging System (Molecular Devices, Sunnyvale, CA.).

The neurospheroids were not disturbed in the flowchips and the flowchip holder is compatible with the FLIPR and high-content imaging systems.

Drug Treatment & Supernatant Sampling

The versatility of the Pu·MA System can be demonstrated by the measurements data acquired for the neurospheroids both from the oscillation kinetic data and subsequently confocal imaging. Figure 3 shows the data acquired from the drug treatment experiments. Firstly analyzing the effect of two different neuro-active compounds on neurospheroids (Gabapentin and Azimilide, Figure 3A). Secondly demonstrating sequential addition of different drug concentrations to a single spheroid to measure changes in calcium oscillations (4-Aminopyridine, Figure 3B). Toxicity effects after drug treatments is shown in Figure 3C, where neurospheroids were stained with cell viability dye calcein AM and high content imaging acquired as previously described (4).

The data shows that using the Pu·MA System you can:

- perform multiple sequential fluid exchanges without damage to the single spheroid
- protocol can be stopped in between for other analyses
- multiple measurements done within the flowchip with minimum perturbation

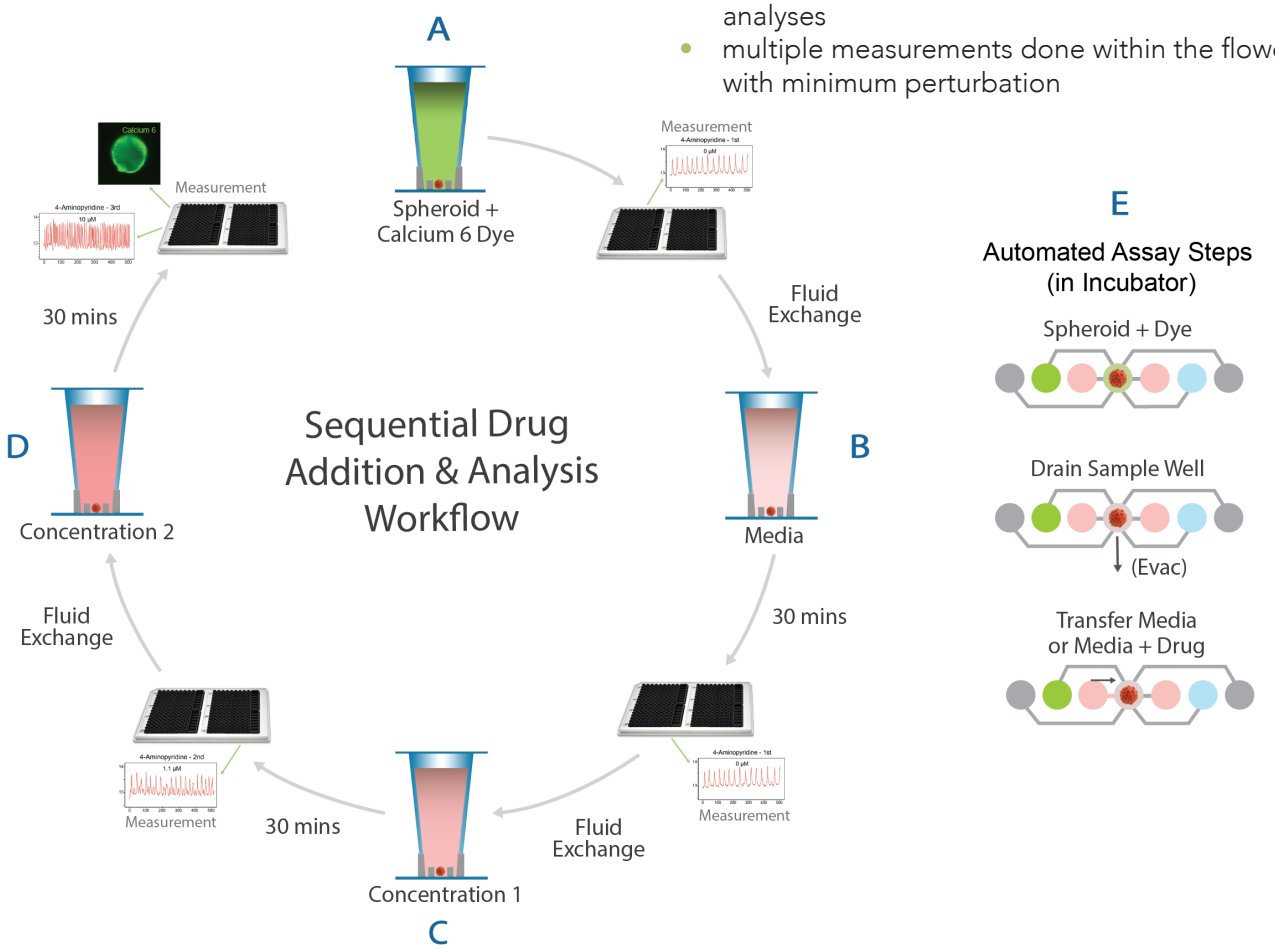

Figure 2. Schematic of sequential drug addition & analysis workflow shows the steps for assaying neurospheroids. All the fluid exchanges were done within the Pu·MA System inside the incubator while Ca^{2+} oscillation measurements were performed with the FLIPR System A The assay begins with neurospheroid incubated with Calcium 6 dye (sensitive to Ca^{2+} oscillations) for 60 mins and then measured for fast kinetic fluorescence imaging and confocal imaging (both denoted in workflow as Measurement). B The media + dye is exchanged for just media, equilibrated for 30 mins and measurement taken before step C where the 1st concentration of drug is added, incubated for 30 mins and measurement taken before step D where the 2nd concentration of drug is added, incubated for 30 mins and measurement taken. Confocal images were acquired using ImageXpress Micro Confocal Imaging System E Schematic of the microfluidic exchanges between wells.

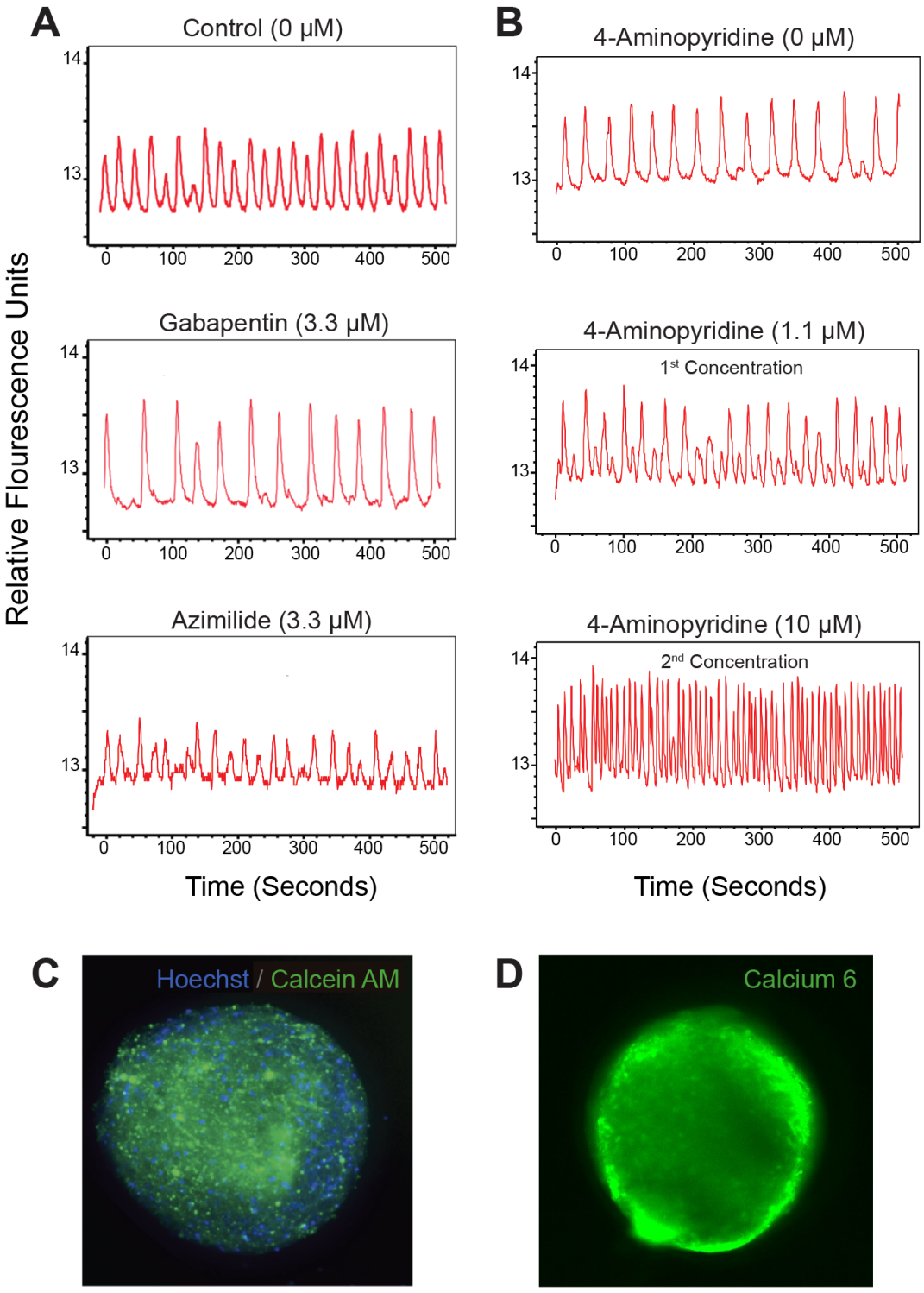

Figure 3. Ca^{2+} oscillations and high content imaging of neurospheroids in in response to drug treatments. A Kinetic fluorescent recording of Ca^{2+} oscillations in measured in response to Gabapentin and Azimilide, by FLIPR Penta High-Throughput Cellular Screening System. Recorded patterns were analyzed, and peaks counted by PeakPro2 software. B Concentration response of a single neurospheroid to sequential 4-Aminopyridine additions. Measurement were taken with media only, then 1st concentration (1.1 µM) after 30 min incubation and then 2nd concentration (10 µM) after an additional 30 min incubation. C Toxicity effects were analyzed by confocal imaging (using ImageXpres Micro Confocal System) of neurospheroids stained with nuclear stain (Hoechst, Blue) and cell viability dye (Calcein AM, Green). D Neurospheroid stained with Calcium 6 dye and imaged at the start of the assay.

Summary

In this application note we have demonstrated capabilities of the Pu·MA System 3D to perform:

- automated neurspheroid processing
- simple user-adjusted protocols
- sequential drug addition and analysis
- *in situ* imaging of cellular activity within flowchips
- multiple readouts from a single sample

Using streamlined workflows and Pu·MA System protocols, we showed staining, imaging and activity measurement of neurospheroids in response to different drugs and drug concentrations.

We showed that with increasing concentration of drug treatments we could quantify phenotypic changes in the cells, such as changes Ca^{2+} oscillations all within physiologically relevant conditions with minimum perturbation to the samples.

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Pu·MA System Microfluidics

The Pu·MA System can automatically perform complex assay steps using proprietary microfluidics. All reagents are loaded into flowchips and then incubation, media exchanges, cell secretion sampling and other steps are executed by the system program (schematic of fluid transfers is shown in Figure 4A).

The key features are:

- 3D cell models remain in the protected sample chamber during the assay (Figure 4B)
- 95% of media can be exchanged without drying cells
- There is no direct fluid flow over cells to disrupt the cell structures (Figure 4B)
- Optically clear bottom allows for high resolution imaging

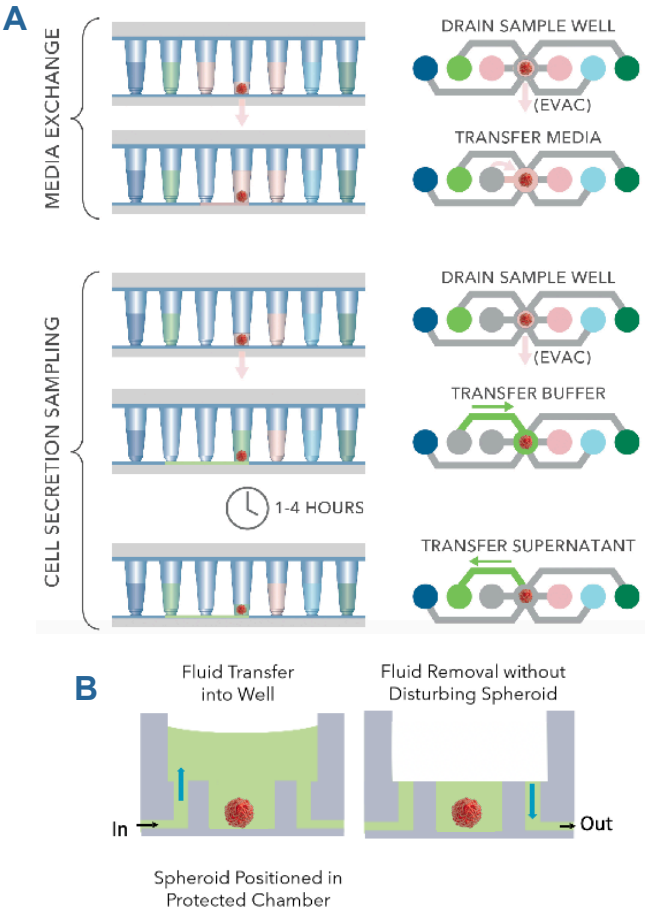

Figure 4. Pu·MA System microfluidics. A Schematic showing typical protocol steps available with the Pu·MA System. All steps are performed automatically inside an incubator. B Schematic of the protected sample chamber and fluid flow.

#3DCellAssay #PuMASystem #flowchip

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