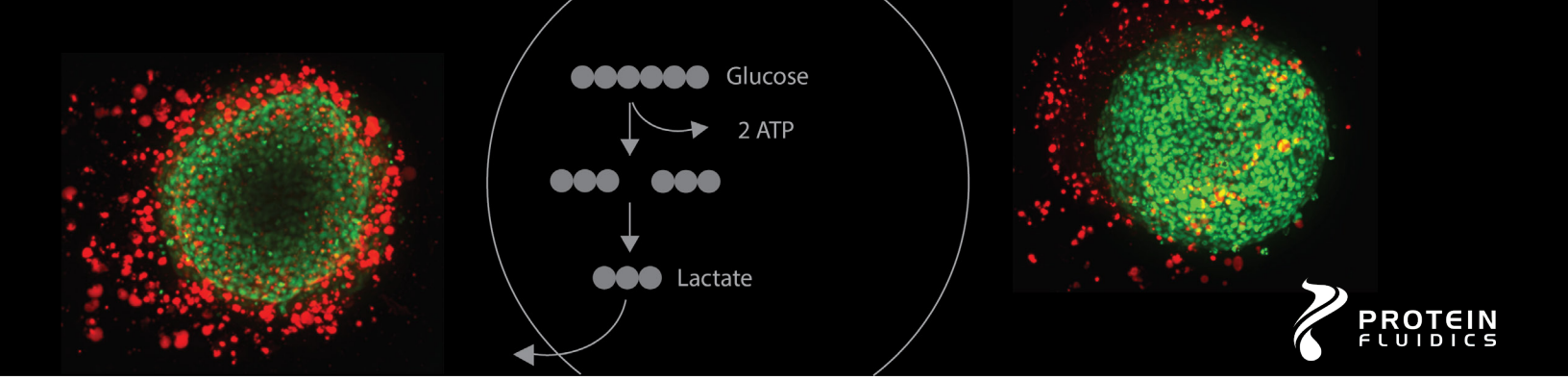


TECHNICAL NOTE

Automated Dynamic *In Situ* Sampling for Metabolite Analysis of Breast Cancer Tumoroids

Introduction

Cancer cells undergo metabolic modifications to meet high bioenergetic and anabolic demand during tumorigenesis. For most tumors, aerobic glycolysis is a major driver of cancer progression, resistance to chemotherapy, and poor patient outcome. Lactate, a product of glycolysis, plays a role in tumor progression and is linked to drug resistance in breast cancer. Patient-derived 3D cancer cell models are valuable tools for research, drug development and personalized medicine, because they recapitulate features of the tumor microenvironment. Therefore, monitoring metabolite dynamics and lactate production in physiologically relevant patient-derived models can provide valuable data for understanding metabolic perturbations during disease progression, drug response and resistance.

In this technote, we demonstrate a workflow to assess metabolic changes in triple negative breast cancer (TNBC) tumoroids in response to treatment with anti-cancer drugs. Our approach utilizes the Pu·MA System with microfluidic flowchips that allows for dynamic *in situ* supernatant sampling, followed by a sensitive luminescence lactate assay and high content imaging.

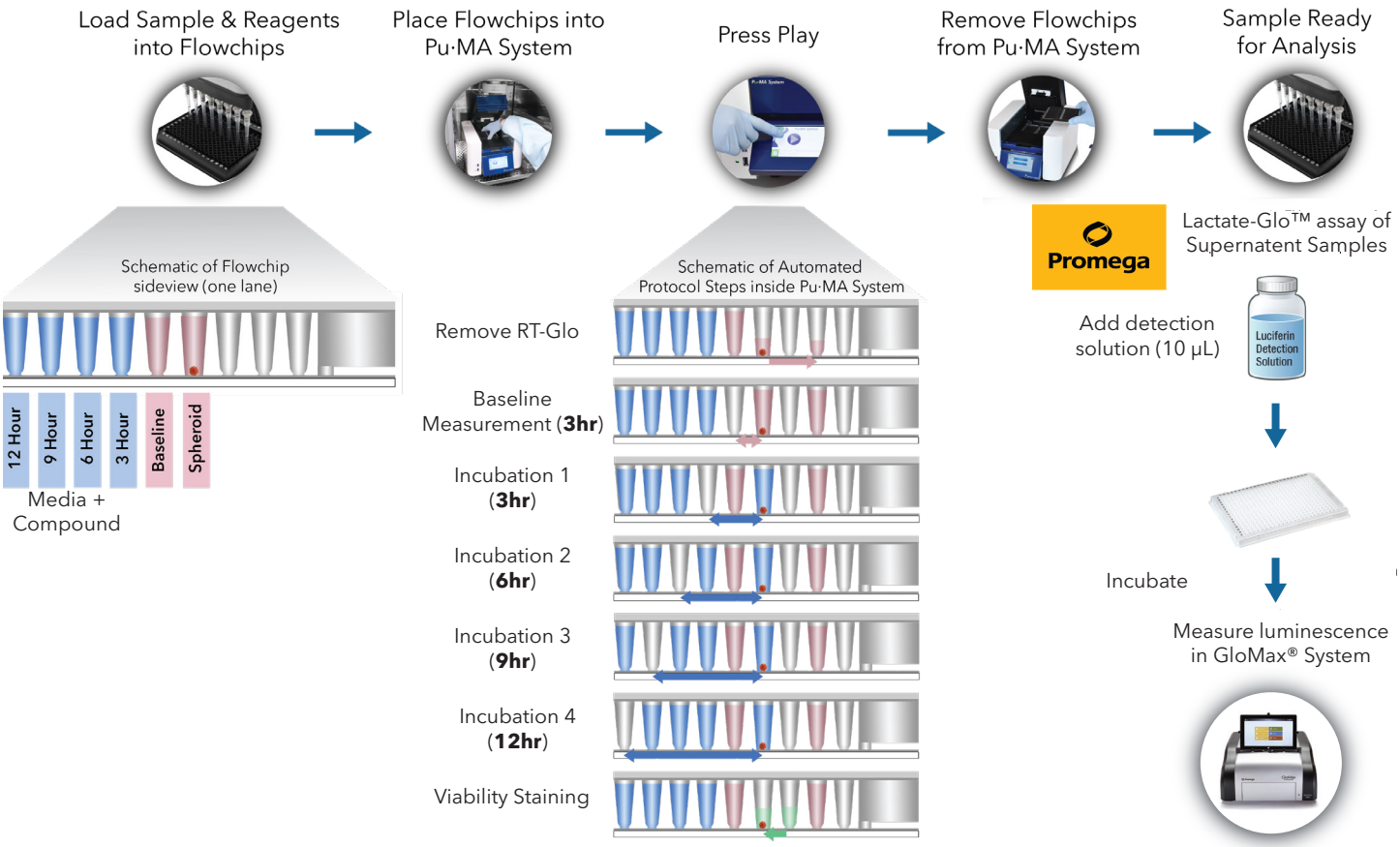


Fig 1. Metabolite secretion assay workflow with schematic representation of automated steps within the Pu·MA System. This is followed by supernatant analysis for the secreted metabolite lactate.

Procedure

Patient-Derived Tumoroid Formation

For this study we used patient-derived TNBC tumoroids (TU-BcX-4IC, abbreviated to 4IC)[1,2]. Tumoroids were created by seeding 2000 – 4000 cells/well into ultra-low attachment 96 or 384 well plates (Fig 2) and culturing for 72 hours using methods described previously[1,2].

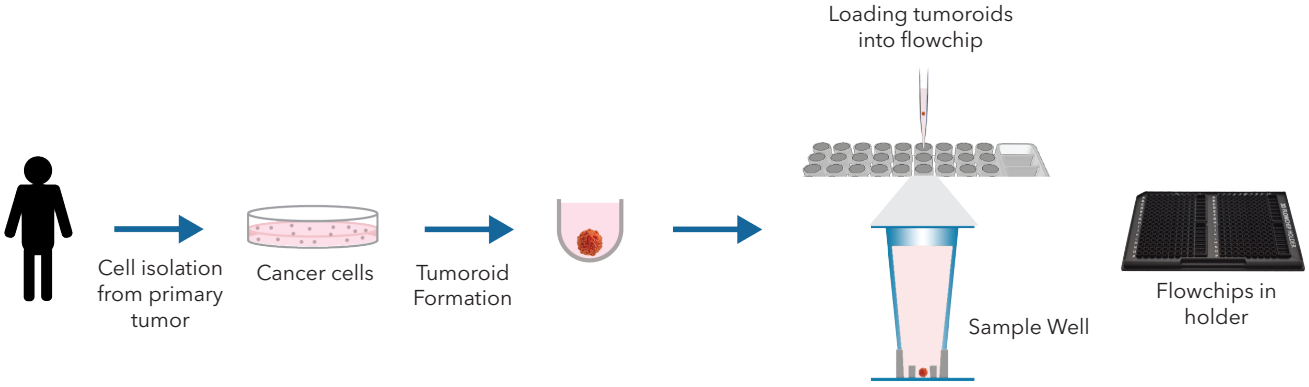


Fig 2. Schematic of tumoroid formation and transfer to Pu·MA System

Note: Tumoroids can be coated with NanoShuttle® magnetic nanoparticles, which can help in transferring and positioning the 3D cell models within the sample well of the flowchip as shown previously[6].

The complete metabolite dynamics assay is performed in 3 parts:

A. Viability and uniformity assessment of the tumoroids (pre-assay)
B. Timed drug treatment and in situ sampling for metabolite analysis (assay)
C. End-point viability staining & confocal imaging (post-assay)

Note: Place Pu·MA System into the incubator for at least 30 mins before the assay for temperature equilibration (if required).

(A) 3D Cell Model Assessment for Viability & Uniformity

To assess tumoroid uniformity and viability before drug treatment, we optimized the RealTime-Glo® assay (Promega, RT-Glo) for 3D cell-based models. The RT-Glo assay continuously monitors cell viability with a luminescence readout proportional to the number of viable cells (Fig 3).

1. 20 µL of RT-Glo reagent was prepared in media.
2. Tumoroids were mixed with RT-Glo media and loaded to the sample well in the flowchips.
3. Samples were incubated for 2 hr at 37°C.
4. Luminescence was measured from flowchip sample wells using a GloMax plate reader.

This pre-assessment assay demonstrated good linearity of the luminescence signal over a range of tumoroid cell density (Fig 3A). The Coefficient of Variation of signal from samples was 26% indicating a high starting uniformity of tumoroid size & viability (Fig 3B). This could potentially be used as a data normalization factor to account for size, number, or viability of tumoroids.

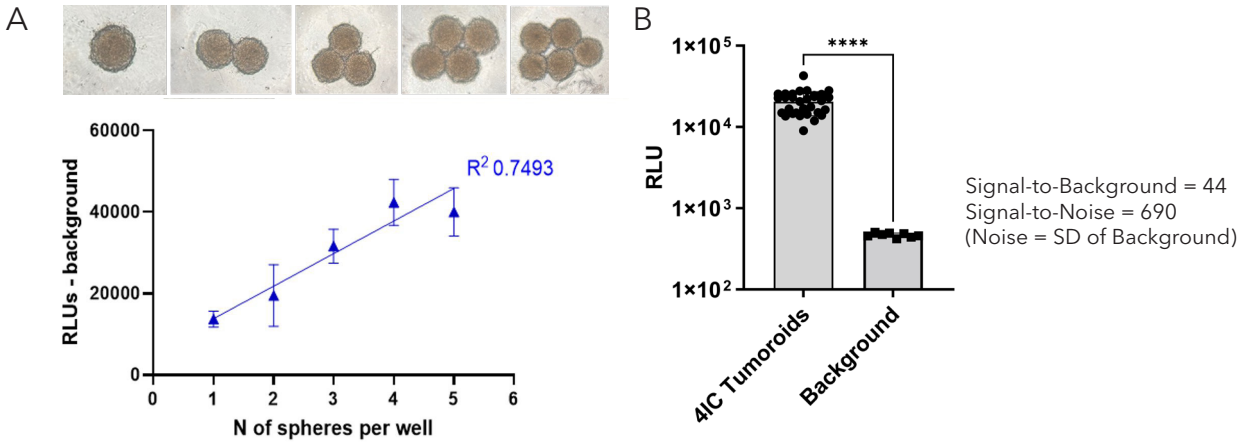


Fig 3. Tumoroid viability and uniformity measured by luminescence using RT-Glo assay within Pu·MA System flowchips.

(B) Timed drug treatment and *in situ* sampling for metabolite analysis

Metabolite secretion of 4IC tumoroids in response to drug treatments was determined by collecting supernatants at five time points over a 12-hour duration in 3-hour intervals as described in the procedure below.

Load Tumoroids and Reagents into the Flowchip

After RT-Glo measurement is completed, load the flowchip with reagents for the drug treatment:

1. Load 20 µL of media to Well 5 (for the baseline pre-treatment secretion measurement)
2. Load 20 µL of media + drug/compound into Wells 1, 2, 3, and 4.
3. Load flowchip holder into the Pu·MA System and on the touchscreen, select and start the pre-designed assay protocol.

Automated Assay Steps

Outlined here are the automated reagent transfer steps executed by the Pu·MA System during the drug treatment and timed *in situ* supernatant sampling protocol (schematic shown in Fig 4).

1. Drain media with RT-Glo from the sample well to Well 8.
2. Transfer media from Well 5 to Well 6.
3. Incubate samples for 3 hr (**baseline measurement**) and then transfer supernatant back to Well 5.
4. Transfer media + drug from Well 4 to Well 6.
5. Incubate sample for 3 hr (**3 hr** point) and then transfer supernatant back to Well 4.

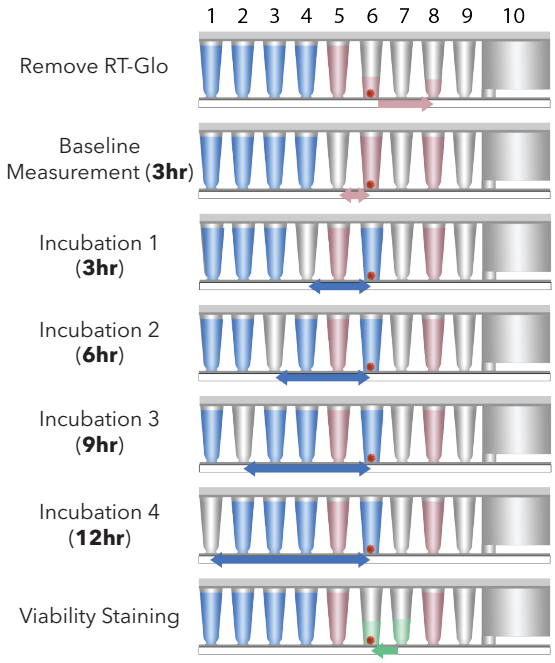


Fig 4. Schematic of automated steps within the Pu·MA Sysytem for collecting timed supernatent samples within flowchips.

6. Transfer media + drug from Well 3 to Well 6.
7. Incubate sample for 3 hr (**6 hr** point) and then transfer supernatant back to Well 3.
8. Transfer media + drug from Well 2 to Well 6.
9. Incubate sample for 3 hr (**9 hr** point) and then transfer supernatant back to Well 2.
10. Transfer media + drug from Well 1 to Well 6.
11. Incubate sample for 3 hr (**12 hr** point) and then transfer supernatant back to Well 1.
12. Remove the flowchips and collect the five stored supernatants samples from Well 1 to 5 in each sample lane. Collected supernatant samples can be stored at -20°C or processed right away for the downstream metabolite measurement.
13. Load the flowchip for viability staining: add 20 µL of dyes for live/dead staining to Well 7.
14. Place holder back into the system and run staining protocol. During this protocol the viability stains solution is transferred from Well 7 to sample well 6, followed by incubation for 3 hours.
15. After the protocol is completed, take out the flowchips and image tumoroids for live & dead cells directly within the flowchips (Fig 6).

Note: We have shown that in this workflow **5 samples** were collected, and an initial real-time viability measurement and final end-point viability assessment by confocal imaging within the flowchip were performed. However, there are 2 more wells available within the flowchip per sample which can be used for additional time points if needed. These reagent transfers can be customized for other assay configurations as well.

Collect Supernatants for Metabolite Analysis

In this experiment the metabolic response of tumoroids to treatment was determined by measuring lactate secretion in collected supernatants using luminescence Lactate-Glo assay. The workflow described in this note can be used for measuring other secreted metabolites and molecules as well.

1. Lactate detection reagent was prepared according to the manufacturer' protocol
2. Supernatant samples were diluted 1:400 in PBS
3. 10 µL of diluted supernatant sample + 10 µL of lactate detection reagent was added into each well of white solid-bottom 384-well assay plate. The plate was incubated for 60 min at room temperature and luminescence was measured with a GloMax plate reader.
4. Figure 5 shows the results for the lactate time course secretion analysis. Tumoroid treatment of over 9 – 12 hr resulted in statistically significant increase in lactate secretion for paclitaxel ($p < 0.05$) and romidepsin ($p < 0.01$) indicating drug-induced shift toward glycolysis.

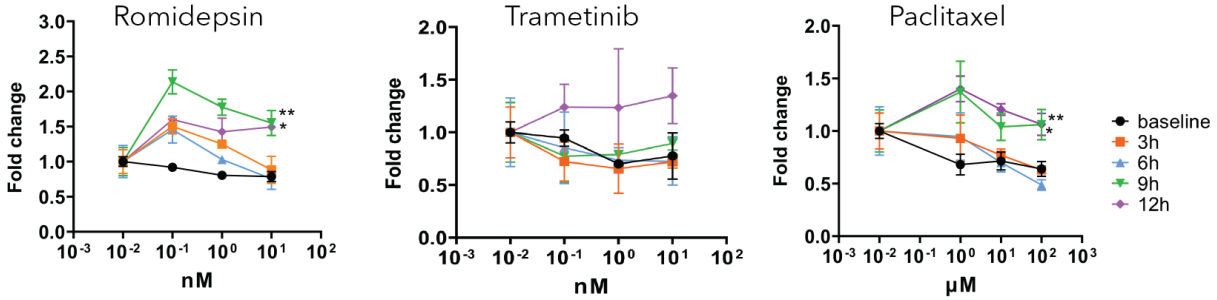
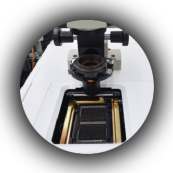


Fig 5. Relative amount of lactate measured in tumoroid supernatant samples as a function of compound concentration and incubation time. Data is shown as fold change relative to the baseline measurement (3 hr incubation with no compound). Each data point represents 3 independent tumoroids. Error bars = +/- 1 SDM.



(C) End-Point Viability Staining & Confocal Imaging

1. Viability staining was done using CyQuant Green (for viable cells) and Ethidium homodimer (EtHD-1, for dead cells) for 3 hr.
2. Tumoroids were imaged within the flowchips using ImageXpress Micro Confocal Imaging system (Fig 6) to acquire z-stacks covering the 3D structures.
3. The images were analyzed by segmenting individual cells and classifying them Live or Dead based on staining intensity. The ratio of live/dead cells in each tumoroid was used to determine effect of drug treatments in comparison to untreated tumoroids.
4. Results showed that viability was maintained in untreated tumoroids after 16 hours. In comparison, tumoroids treated with different drugs showed an increase in number of dead cells. (Fig 6). This data has been published[6].

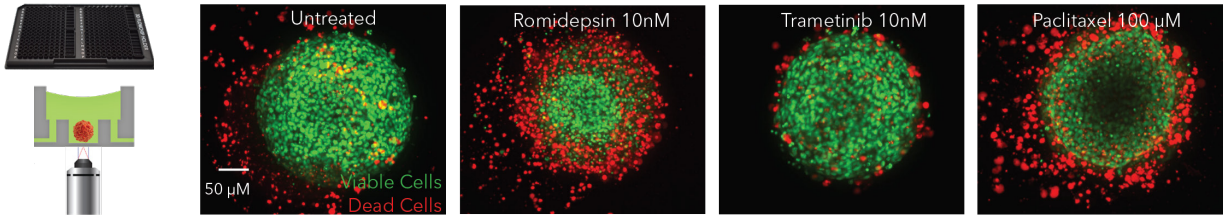


Fig 6. Fluorescent images of 4IC tumoroids treated with different compounds and compound concentrations but with the same treatment duration of 16 hours.

Summary

This tech note demonstrates the capability of Pu·MA System to perform automated dynamic *in situ* sampling of tumoroid supernatants while assaying effects of drug treatments. We have shown that the time-consuming steps of supernatant sampling can be done in a hands-off workflow without perturbing the tumoroids. We have also shown use of the RealTime-Glo assay for 3D cell models which can be performed as a normalization for tumoroid size and viability. We used dynamic supernatant collection to examine effect of drug treatments on production of the important metabolite lactate. This is critical to understanding changes in tumoroid metabolism in response to therapeutic assault. Other metabolites or secreted factors can also be assayed in this manner. For example, in a previous publication we analyzed for changes in VEGF secretion from HepG2 spheroids by measuring supernatant samples with ELISA[3].

A key advantage of the Pu·MA System is multi-functional profiling. In this case tumoroids were further analyzed for viability within the system to characterize their response more completely to drug treatments. This completes the whole story from tumoroid assessment with RealTime-Glo assay before the drug treatment, followed by timed drug-treatment and sampling, followed by staining and imaging of tumoroids within the Pu·MA System flowchips. The Pu·MA System protocols are customizable and can be optimized based on the experimental aim.

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- Molecular Devices, Inc.: Dr. Oksana Sirenko & Mathew Hammer
- Promega, Inc.: Dr. Jolanta Vidugiriene, Donna Leippe, Dr. Terry Riss & Kim Haupt

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Materials

Patient-derived tumoroids – 4IC (as published previously)[1,2,6]
Ultra-Low Attachment 96 well plate (Corning Costar #CLS7007)
Matrigel (Corning #356234)
PBS
NanoShuttle (Greiner Bio-One)
Lactate-Glo Assay (Promega)
RealTime-Glo MT (Promega)
Romidepsin
Trametinib
Paclitaxel
Pu·MA System (Protein Fluidics)
Pu·MA System 3D Flowchips (Protein Fluidics)
ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices)
GloMax-Multi+ Detection System with Instinct Software (Promega)

Instrument Compatibility

Light microscope
Automated Confocal Imaging Systems or any other fluorescence microscopes
Plate Reader (384 well spacing)

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