APPLICATION NOTE

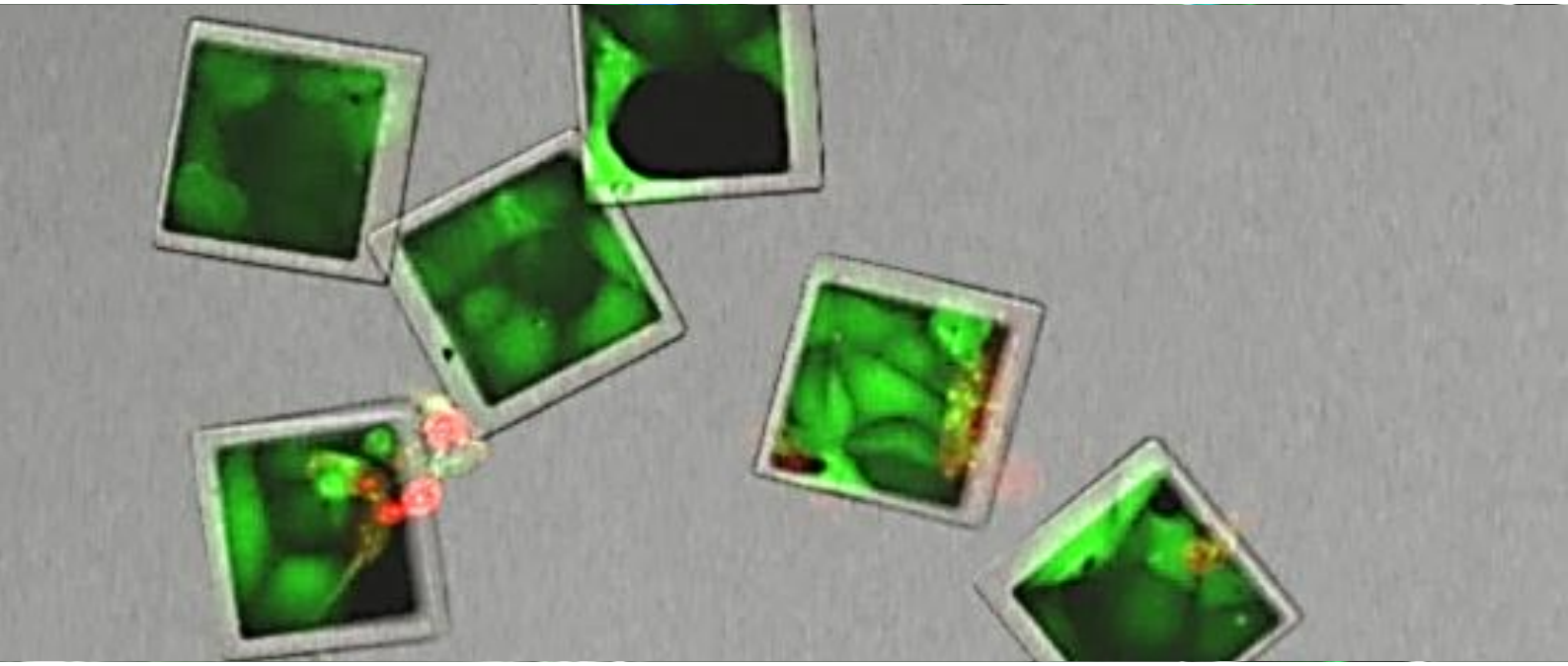
SemaCyte® Microcarriers for Assay-Ready Adherent Cells: Rapid Dose-Response Screening on Human Kidney Cancer Cells

Jeroen Verheyen[1], Tannia Gracia[2], Tim Fitzmaurice[3], and Tarun Vemulkar[1]

(1) Semarion Ltd, UK
(2) Milner Therapeutics Institute, University of Cambridge, UK
(3) Biological and Soft Systems Sector of the Cavendish Laboratory (BSS), University of Cambridge, UK

Abstract

Assay-ready cells are reagents used for instant assaying. While successful for suspension cells, adopting this assay-ready format for adherent cells has been challenging due to their need to attach to a surface such as that of a well-plate. In this study we demonstrate how SemaCyte® cell assaying microcarriers are uniquely positioned to overcome this limitation. SemaCyte® Seeding Discs were loaded with RCC-FG2 human kidney cancer cells to generate vials of frozen adherent cells. To evaluate the reliability and reproducibility of the SemaCyte® assay-ready adherent cells, an ionomycin dose-response experiment was performed at 3 separate centres by different users. Results showed that the EC50 were consistent between the centres and comparable to the controls. Using the SemaCyte® assay-ready approach the total experimental time was cut in half and the number of cells per assay was reduced by over 4-fold.



Introduction

Assay-ready or frozen instant cells are those cryopreserved at a highly functional state. Like other reagents, these cells can be thawed and instantly applied to a cell-based assay without prior cultivation or passaging. While initially used to facilitate rapid assays in drug screening campaigns, this method has gained a broader scope of applications.

Currently, the selection of cell types is limited to those which are suspension-based. Applications have primarily focussed on binding or pathway activation assays performed on cells such as HEK293 or leukocytes. As adherent cells are used predominantly in drug screening workflows and other life science research, this limitation to suspension-based cells presents a roadblock for more widespread adoption of assay-ready cell formats.

While adherent cells can be stripped and frozen, they typically need time to attach and regain their native morphology after plating. This makes it challenging to adopt them as assay-ready cells. While it is possible to freeze full culture plates with adherent cells, this would take up large amounts of freezer space and eliminate the reagent-like nature of assay-ready cells.

Semarion's SemaCyte® microcarriers are uniquely able to overcome these limitations. Adherent cells can be seeded on SemaCyte® Seeding Discs in large batches (**Figure 1**). After the correct morphology and confluency is achieved, cell-containing SemaCytes are lifted of into suspension and frozen as assay-ready adherent cell batches. To perform a rapid assay, cell-containing SemaCytes are thawed and plated for instant assaying. Throughout this process, the cells retain their morphology and local confluency.

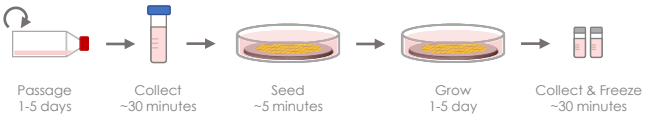


Figure 1. Generation of SemaCyte® Assay-Ready Adherent Cells. *After passaging, the cells of interest are loaded at a desired density on SemaCyte® Seeding Discs. After the desired confluency and morphology is reached, cell-containing SemaCyte® microcarriers are released into suspension and frozen as assay-ready reagents,*

Here, we demonstrate the reliability and reproducibility of the SemaCyte® assay-ready adherent cells by performing a rapid ionomycin dose-response assay. A batch of RCC-FG2 assay-ready human kidney cancer cells was produced using SemaCytes. Three different centres performed plate-reader based viability assays using a different vial and compared the outputs to a concurrent control experiment (**Figure 2**).

Materials and Methods

RCC-FG2 cells (passage 25) were seeded onto 2-inch SemaCyte® Seeding Discs at a density of 50,000 cells/cm^2. Cells were incubated for 24 h in culture medium, namely RPMI-1640 (Gibco) supplemented with foetal calf serum, glutamine, pyruvate, and pen-strep. SemaCyte® Seeding Discs were agitated to release cell-loaded SemaCytes. After magnetic separation, the microcarriers were resuspended in cryopreservation medium. Two aliquots of 0.5 mL were frozen at a rate of 1°C/min and stored at -70°C (**Figure 1**). One aliquot provides enough SemaCytes for half a 96-well plate at about 400 microcarriers per well.

To perform a rapid ionomycin dose-response assay, an aliquot of the SemaCytes assay-ready adherent cells were snap thawed. The SemaCytes were moved to 5 mL culture medium using magnetic separation. They were plated into rows A-D of a 96-well plate using a magnetic plate holder. After 1 h of incubation, a serial dilution of ionomycin (Merck Millipore) in DMSO was added to each row of the plate, in quadruplicate. After 24 h, CellTiter-Fluor™ (Promega) was incubated for 1 h. The cell viability was assessed with a fluorescent plate reader (BMG Labtech CLARIOstar PLUS or FLUOstar Omega) (**Figure 2**).

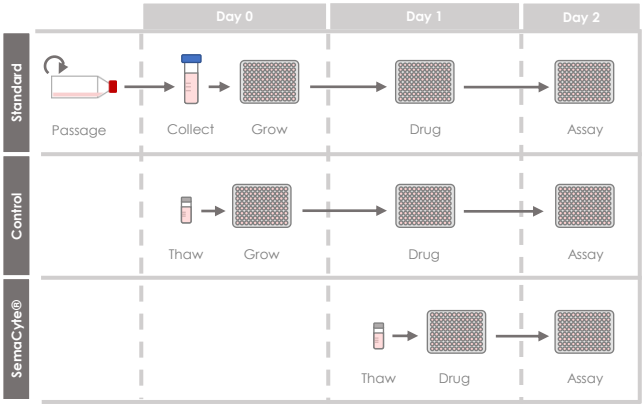


Figure 2. Diagram of Dose-Response Assays. *A typical protocol (Standard), an assay-ready suspension cells protocol (Control), and an assay-ready adherent cells protocol (SemaCyte®) are overviewed. In this study, ionomycin was incubated with RCC-FG2 cells for a period of 24h.*

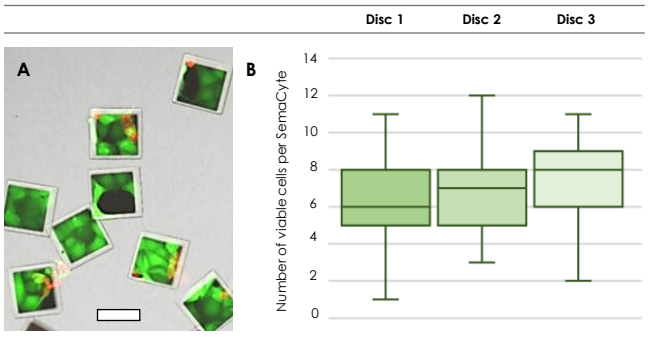
As control, RCC-FG2 cells from the same passage as above were frozen into separate aliquots as suspension cells. One aliquot provided enough cells to seed half a 96-well plate at a density of 50,000 cells/cm^2. These suspension cells were snap thawed, centrifuged, and plated into rows E-H 24 h before ionomycin was added (**Figure 2**).

SemaCytes and control cells were stained at various stages of the rapid assay using a LIVE/DEAD™ Viability/Cytotoxicity Kit (ThermoFisher Scientific). Using live cell imaging (Leica TCS SP5 with 10x objective) the viability, confluence, and morphology was further analysed. Semarion's image analysis script was used to digitally isolate SemaCytes for downstream image quantification.

Loading SemaCyte® Seeding Discs

Three 2-inch SemaCyte® Seeding Discs were loaded with RCC-FG2 cells at a density of 50,000 cells/cm^2. After 24 h of attachment, SemaCytes were released into liquid and cryopreserved as assay-ready adherent cells (**Figure 1**).

Samples from each Seeding Disc were plated into a 96-well plate to analyse viability, confluency, and morphology after microcarrier release using live cell imaging (**Figure 3**). An average of 38 ± 6 SemaCytes per well were imaged and analysed. For each of the three separately loaded SemaCyte® Seeding Discs, the cell numbers, viability, and area coverage of the released SemaCytes were comparable (**Figure 3**). The average number of AM-Calcein positive (viable) cells per 0.01 mm^2 SemaCyte® was 6.8 ± 0.4. The average cellviability, measured as AM-Calcein positive (viable) vs EthD1 positive (dead) cells, was 85% ± 1%. The average AM-Calcein positive area coverage (confluency) was 71% ± 5%.



C	Disc 1	Disc 2	Disc 3
Number (n) of SemaCytes imaged	49	27	39
Number of viable cells	6.2 ± 0.36	7.0 ± 0.44	7.3 ± 0.40
Cell viability	86% ± 2.3%	86% ± 1.8%	84% ± 2.4%
Viable cell area coverage	64% ± 2.7%	69% ± 3.2%	80% ± 2.8%

Figure 3. Loading and Release of SemaCytes. *The loading reliability and efficiency of RCC-FG2 cells onto three separate SemaCyte® Seeding Discs was compared. (A) After 24 h cell attachment, SemaCytes were released and stained with AM-Calcein (viable, green) and EthD1 (dead, red). Scale bar is 100 µm. (B) The box plot show the distribution of the number of live cells per carrier released from the three Seeding Discs. (C) The table further summarizes key cell loading parameters for each Seeding Disc. Values are shown as average per carrier ± SEM.*

Rapid Dose-Response Assays on Adherent Cells

To evaluate the reliability and reproducibility of the SemaCyte® assay-ready adherent cells, an ionomycin dose-response experiment was performed by different users at 3 centres, namely at Semarion, the Milner Therapeutics Institute, and the Biological and Soft Systems Group of the Cavendish Laboratory at the University of Cambridge. Each centre used a vial of frozen SemaCytes loaded with assay-ready RCC-FG2 cells and a vial of control suspension cells, all from the same batch.

Control suspension cells were thawed and plated on day 0 to allow them to attach and obtain an adherent morphology. They were loaded at a density of 50,000 cells/cm^2 to mimic the SemaCyte® loading protocol described above. On day 1, SemaCyte® assay-ready adherent cells were thawed and plated. A serial dilution of ionomycin was added to both the SemaCytes and the control cells in quadruplicate. On day 2, the fluorescent viability dye was added to evaluate the ionomycin-induced cell death of RCC-FG2 cells (**Figure 2**).

The ionomycin dose-response curves produced at each centre were comparable (**Figure 4**). Both the SemaCytes and control conditions yielded similar results. An asymmetric sigmoidal 5PL regression was used to calculate an EC50 and a goodness of fit. The average EC50 for ionomycin-induced killing of RCC-FG2 cells was 2.34 µM ± 0.43 µM and 2.40 µM ± 0.38 µM for SemaCytes and controls, respectively. The R squared for the goodness of fit was 95-98% for SemaCytes and 97-99% for controls.

SemaCytes were titrated into the 96-well plate at about 400 microcarriers per well, covering 10-15% of the well. The differential fluorescence intensity between SemaCytes and control wells in the 0 µM ionomycin conditions confirmed that an average of approximately ~4.7 times less cells were present in the SemaCyte® wells compared to the control wells.

Altogether, this showed that the SemaCyte® assay-ready adherent cells were able to produce reliable and reproducible dose-response data, while cutting the total experimental time in half and reducing the number of cells used per well by over 4-fold.

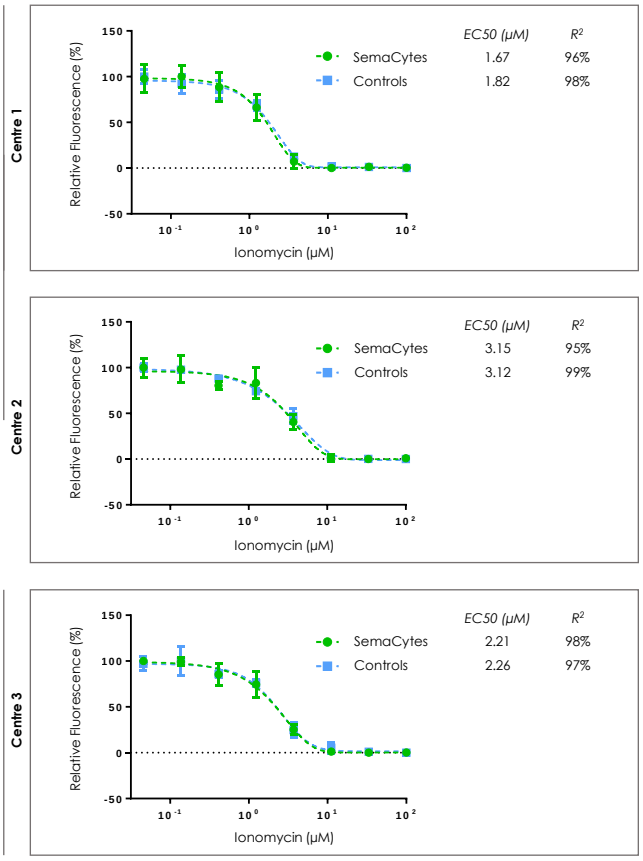


Figure 4. Ionomycin Dose-Response Assay on RCC-FG2 Cells Across Multiple Centres. *Assay-ready suspension cells (controls, blue) and assay-ready adherent cells (SemaCytes, green) from the same batch were deployed for a rapid assay using a 2-day and 1-day protocol, respectively. After 24 h of incubation with an ionomycin serial dilution in quadruplicate, cell viability was analysed on a plate reader with CellTitre-Fluor™. EC50 and R squared values were calculated using a 5PL regression (dotted line).*

Microscopy Analysis of Assay-Ready Adherent Cells

To further evaluate the viability, confluence, and morphology of RCC-FG2 cells frozen on SemaCytes, cells were labelled with AM-Calcein and Ethidium Bromide D1 to stain live and dead cells, respectively. Imaging was performed during each step of the protocol used for the dose-response assay, for both the SemaCyte® and control condition (**Figure 5-6**).

Individual SemaCytes were digitally isolated for downstream quantification (**Figure 5**). For the controls, full images were used. The numbers of live and dead cells and the area of live cell coverage were measured using an image analysis script (**Figure 6**). The number of live cells per 0.01 cm^2 on day 1 was 6.1 for SemaCytes and 3.8 for controls. This increased to 7.1 and 4.5 on day 2, correlating to a 16% and 18% increase for the SemaCytes and controls, respectively. Between day 1 and 2 the confluency increased from 63% to 86% for SemaCytes and from 38% to 64% for controls.

While all cell seeding was done at 50,000 cells/cm^2, for both the SemaCyte® Seeding Discs and the control wells, the measured cell density and confluency was higher on the SemaCytes. This could be attributed to Semarion's proprietary cell attachment polymer which was used on the SemaCytes. Previous experiments have shown quicker cell capture and faster cell growth when comparing

attachment to this polymer vs untreated well plates. For the controls, untreated wells were used. Further, the trypsinization prior to freezing of the control cells might also contribute to the observed difference in growth and coverage.

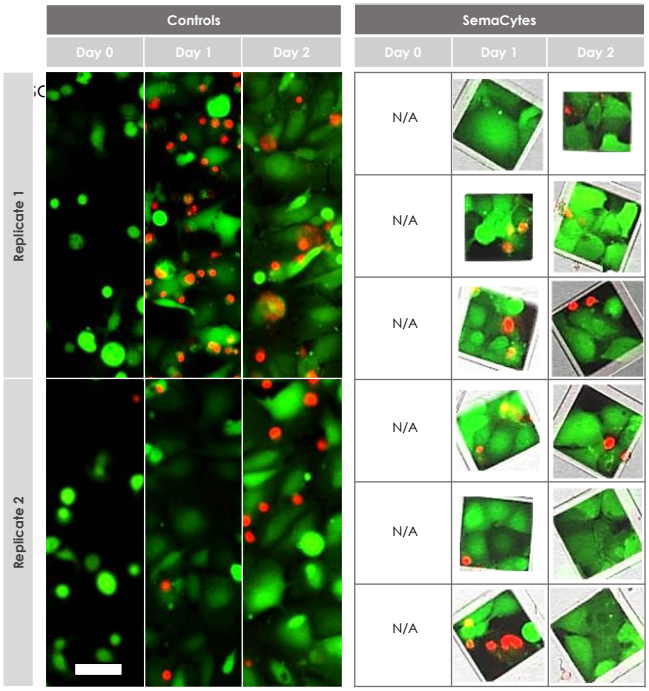


Figure 5. Microscopy Images of RCC-FG2 Cells at Various Stages of the Rapid Assay Protocol. *For both the SemaCyte® and control protocol, untreated (0 µm ionomycin) cells were stained with AM-Calcein (live, green) and EthD1 (dead, red) on day 0 (thawing suspension cells), day 1 (thawing SemaCytes), and day 2 (24 h dose-response incubation). Control images were cropped to the centre. Digitally isolated SemaCyte® images were chosen at random. Scale bar: 100 µm.*

Figure 6. Analysis of Cell Density and Confluency of RCC-FG2 Cells at Various Stages of the Rapid Assay Protocol. *An image analysis script was used to measure (top) the amount of live (AM-Calcein positive) and dead (EthD1 positive) cells, and (bottom) the area of live (AM-Calcein positive) cells. Cell numbers are represented per 0.01 mm², equal to the area of one SemaCyte®. For controls, the whole images were analysed. For SemaCytes, digitally isolated carriers were analysed and averaged. Error bar: SEM across two replicates.*

Conclusion

Overall, this study highlights that SemaCytes can be used to generate reliable and reproducible assay-ready adherent cells. In this design specifically, human kidney cancer cells were successfully loaded onto SemaCytes and frozen in their adherent state for later use in rapid dose-response assays. Values such as EC50 were consistent between various users and between the SemaCytes and controls. While more than 4 times less cells were used per well for the SemaCytes condition, the local cell density and confluency was higher for the SemaCytes than controls. By deploying assay-ready adherent cells, the experimental time for this specific rapid assay was reduced from 48 hours to 24 hours.

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For further information

E: info@semarion.com
W: www.semarion.com

Semarion Ltd
Cavendish Laboratory
University of Cambridge