APPLICATION NOTE

SemaCyte® Microcarriers as Assay-Ready Seeding Shuttles for Multi-Day Drug Sensitivity Assays

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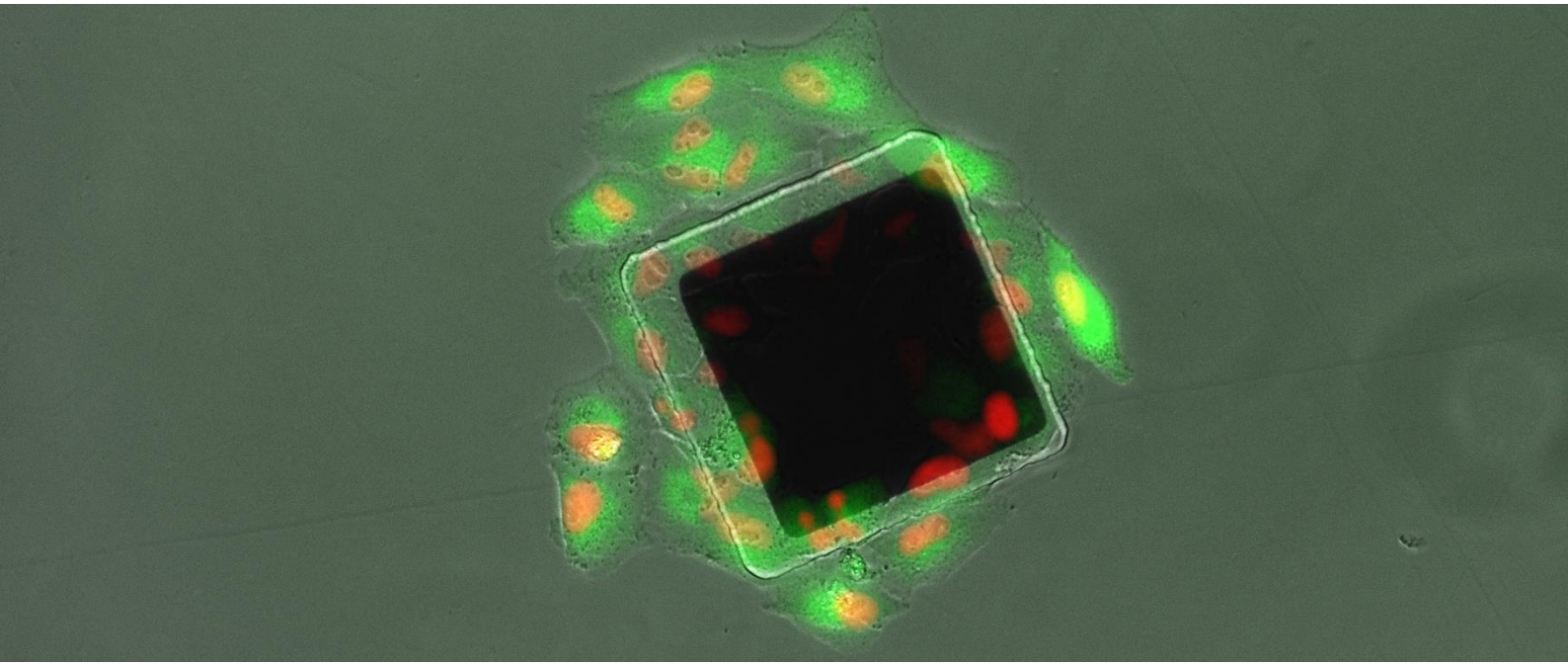
Abstract

Multi-day drug sensitivity assays are commonly used in drug discovery and oncology research where cells are allowed to go through multiple cell divisions in the presence of therapeutic compounds. These assays normally require continuous sub-culturing and preparation of cells, requiring time and reagents. In this study, we demonstrate how SemaCyte® microcarriers are uniquely positioned to overcome these limitations. SemaCyte® Seeding Dishes loaded with A549 human lung cancer cells were frozen and, immediately after thawing, used as seeding shuttles for proliferation assays. SemaCytes produced assays of excellent quality (Z' factor 0.7) and were used to evaluate the potency of targeted therapies such as Nutlin-3A (E3 ligase inhibitor) and Trametinib (kinase inhibitor). The IC50 values obtained for these compounds were comparable to standard assays whilst reducing assay time and increasing assay flexibility.



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.

In our application note "SemaCyte® Microcarriers for Assay-Ready Adherent Cells: Rapid Dose-Response Screening on Human Kidney Cancer Cells" we demonstrated the reliability and reproducibility of SemaCyte® assay-ready adherent cells in a typical 24-hour drug response experiments such as viability assays. In these experiments, the SemaCyte® assay-ready approach cut assay time in half and reduced cell usage by over 4-fold.

Multi-day drug assays are common in oncology research, particularly in the lead optimization and profiling phases of the drug discovery pipeline. These assays allow for cells to go through the multiple cell divisions as required for many therapies undergoing preclinical investigation, for example, to study the effects on cancer cell proliferation. Cell preparation for multi-day assays involves either continuous culturing or deploying ready-to-use frozen stocks at the time of assay (**Figure 1**). The first approach requires significant time and material resources and may increase inter-assay variability due to genetic drift of the cultured cells. These limitations are somewhat addressed by the second approach. However, the need for post-thaw recovery of suspension cells, including to attain a desired adhered morphology, still introduces assay delays and potential variability. Both approaches also require cell trypsinisation prior to dispensing cells for assaying, potentially introducing additional artefacts.

Here, we successfully use SemaCyte® Microcarriers as seeding shuttles for 72-hour proliferation assays, reducing the need for continuous culture or recovery of frozen cells, enabling time and resource savings and greater flexibility in running these assays.

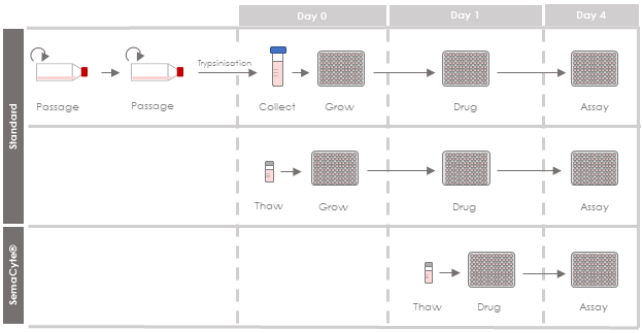


Figure 1. Diagram of a 72 hour drug assay. *Two typical protocols (Standard) and an assay-ready adherent cell protocol (SemaCyte®) are overviewed.*

Materials and Methods

A549 cells were seeded onto a 2-inch SemaCyte® Seeding DIsh at a density of 40,000 cells/cm^2. Cells were incubated for 24 h in RPMI-1640 (Gibco)culture medium supplemented with foetal calf serum, glutamine, pyruvate, and pen-strep. SemaCyte® Seeding Dishes 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 -80°C. One aliquot provides enough SemaCytes for a 96-well plate at about 600 microcarriers/cm^2. SemaCyte® loading and post-thaw recovery was assessed by staining with AM-Calcein (ThermoFisher Scientific), HCS Nuclear Mask Deep Red (ThermoFisher Scientific) and ethidium homodimer (ThermoFisher Scientific). Cells were imaged using an EVOS microscope (10x objective).

For drug assays, standard control cells were passaged until ready to use and seeded into 96-well plates at a density of 2,500 cells/cm^2. Drugs were then added at the indicated concentrations the next day. For SemaCytes, frozen stocks were thawed and allowed to recover for 30 minutes before plating into 96-well plates at a density of 600 microcarriers/cm^2. Drug was added at the indicated concentrations immediately after plating. Cell viability was quantified using CellTiter-Glo 2.0 (CTG, Promega) according to manufacturers specifications using a BMG Labtech FLUOstar Omega plate reader.

Reproducible Loading of A549 onto SemaCyte® Seeding Dishes

A549 is a non small cell lung cancer (NSLC) cell line commonly used in cancer research (>3,500 citations in 2021). To check their compatibility with SemaCytes, two different users loaded four 2-inch SemaCyte® Seeding Dishes with A549 cells at a density of 40,000 cells/cm^2. After 24 hours of attachment, SemaCytes were released into suspension and samples from each Seeding Dish were plated into a 96-well plate to analyse viability (**Figure 2**). An average of 30 SemaCytes per well were imaged and analysed. For each of the four separately loaded SemaCyte® Seeding Dishes the viable cell numbers of the released SemaCytes were comparable (**Figure 2**). The average number of AM-Calcein positive (viable) cells per SemaCyte® was 10.8 ± 0.7.

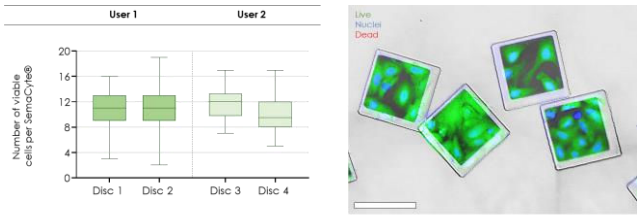


Figure 2. Analysis of SemaCyte® loading with A549. *Four 2-inch SemaCyte® Seeding Dishes were seeded with A549 cells at 40K cells/cm^2. After 24h incubation, SemaCytes were released into suspension and magnetically purified. The box plots show the number of viable cells (AM-Calcein positive) on SemaCytes produced by different users. Representative images of A549 on SemaCytes are shown. Scale bars are 100 µm.*

High freeze-thaw recovery of A549 loaded SemaCytes®

SemaCytes produced as described above and stored at -80°C were assessed for cell viability post-thaw. Although a slight reduction in viable cells per SemaCyte® was observed post-thaw, SemaCytes still had a mean viability of >90% (**Figure 3**). For assay-ready cells, a more important post-thaw metric is recovery. This metric represents the proportion of cells frozen that recover post-thaw and we observed a 94% recovery with A549 loaded SemaCytes. This demonstrates that A549 cells loaded onto SemaCytes maintain a high level of viability directly after thawing making them suitable for use in assays within an hour post-thaw.

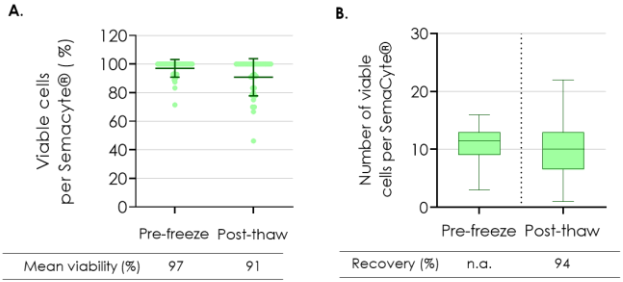


Figure 3. Analysis of thawing of A549 loaded SemaCytes. A. *Viable cells (Calcien-AM positive) and dead cells (Ethidium homodimer positive) per SemaCyte® were quantified post-thaw and the percentage of viable cells per SemaCyte® is displayed. Each point represents a single SemaCyte®.* ***B.*** *Box plots show the number of viable cells (AM-Calcein positive) on SemaCytes. Recovery (mean viable cells per SemaCyte® post-freeze/ mean viable cells per SemaCyte® pre-freeze) is indicated.*

SemaCytes as seeding shuttles yield robust viability and drug potency assays

CellTiter-Glo™ (CTG) is a commonly used luminescent assay that detects cellular ATP and is used to assess cell viability in drug assays. CTG luminescence showed a linear relationship with the number of A549 loaded SemaCytes per well immediately after dispensing (R^2 = 98%, **Figure 4**). This highlights high compatibility between CTG and SemaCytes, alongside the potential dispense specific numbers of SemaCytes into wells.

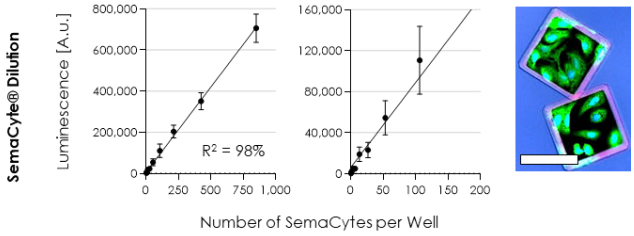


Figure 4. Compatibility of SemaCytes with CellTiter-Glo 2.0. *A serial dilution of A459-loaded SemaCytes was performed in a 96-well plate and assayed with CellTiter-Glo™. The image shows staining with AM-Calcein (live, green), HCS NuclearMask (nucleus, blue), and ethidium homodimer-1 (dead, red). Scale bars are 100 µm.*

We evaluated the ability of SemaCytes to act as seeding shuttles for long-term viability assays. The SemaCytes had a high local confluency and were dispensed as colonies from which cells could grow onto the microwell plate. Z' factor metric assessment was performed to analyse assay quality. Specifically, negative control (vehicle, DMSO) and positive control (drug, 50 µM ionomycin) was assessed using CTG at multiple timepoints. Assays with a Z' factor >0.5 are considered excellent quality. When we used SemaCytes as seeding shuttles we produced assays with Z' factors of 0.87 and 0.74 for 48 hour and 120 hour assays, respectively (**Figure 5**). This demonstrates that the SemaCytes can deliver robust assay quality for viability assays up to and including 120 hours in duration.

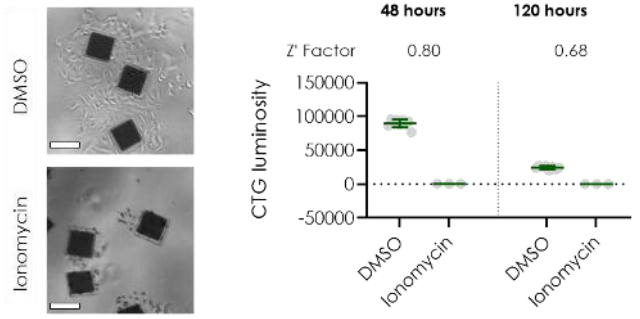


Figure 5. A549 Loaded SemaCytes as seeding shuttles for proliferation assays. *SemaCytes were seeded into well plates and cell viability was assessed 48 and 120 hours after seeding using CTG2.0. Left, images taken at 48 hours post seeding show cellular outgrowth in DMSO controls but not in cells treated with 50µM ionomycin. Right, CTG values across multiple DMSO or ionomycin treated wells after 48 or 120 hours of growth. Dots show values from individual wells and green bars represent mean and standard deviation. Scale bars are 100 µm.*

Batches of adherent assay-ready A549 cells were then used for 72 hour proliferation assays to evaluate the potency of two targeted therapies, Nutlin-3A (E3 ligase inhibitor) and Trametinib (kinase inhibitor). Drug curves produced with A549 loaded SemaCytes were compared with a 'Standard' control where cells are maintained in culture and then seeded and left to attach for 24 hours prior to drugging (**Figure 6**). Drugging of cells on SemaCytes was performed within 60 minutes of thawing.

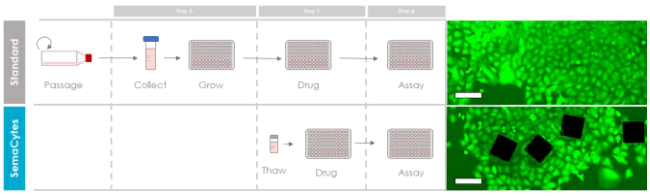


Figure 6. Diagram of A549 Proliferation Assay. *A basic passage-and-use assay workflow (Standard) was compared to an adherent assay-ready cell workflow (SemaCytes). The fluorescence images show untreated cells at the end of the protocol, stained with AM-Calcein (live, green). Scale bars are 100 µm.*

For both Nutlin-3A and Trametinib, SemaCytes and Standard assays produced drug sensitivity curves with comparable IC50 and R^2 values (**Figures 7 and 8**). This data indicated that SemaCytes can be deployed for 72-hour proliferation assays to elucidate drug sensitivity.

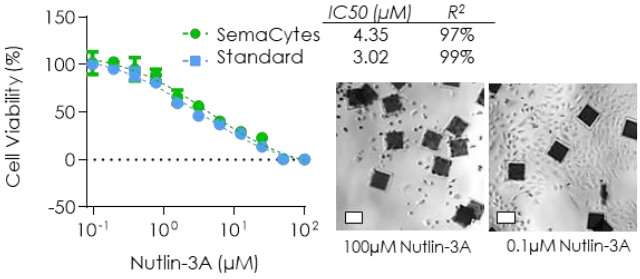


Figure 7. Nutlin-3A sensitivity of A549 cells. *(Standard) A549 cells were passaged, detached and dissociated, seeded at 2.5K/cm², and grown for 24 hours prior to drugging. (SemaCytes) Assay-ready adherent A549 cells on SemaCytes were thawed and dispensed at 600 SemaCytes/cm². For both conditions. Nutlin-3A was incubated for 72 hours and CellTiter-Glo 2.0 (Promega) was used to measure cell viability. Mean and standard deviation of triplicate wells are shown. Scale bars are 100 µm.*

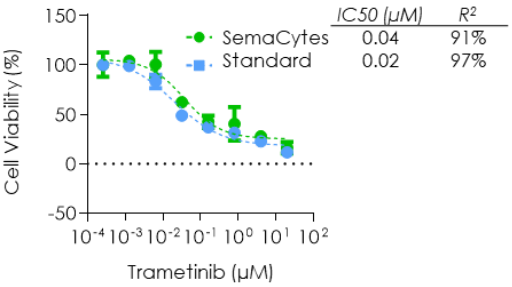


Figure 8. Trametinib sensitivity of A549 cells. *(Standard) A549 cells were passaged, detached and dissociated, seeded at 2.5K/cm², and grown for 24 hours prior to drugging. (SemaCytes) Assay-ready adherent A549 cells on SemaCytes were thawed and dispensed at density of 600 SemaCytes/cm². For both conditions. Trametinib was incubated for 72 hours and CellTiter-Glo 2.0 (Promega) was used to measure cell viability. Mean and standard deviation of triplicate wells are shown.*

Conclusion

Here, we demonstrate the use of A549 loaded SemaCytes as seeding shuttles that can be used in drug viability and drug potency assays directly after thawing. SemaCyte® seeding shuttles delivered assays with excellent quality (Z' >0.7) whilst giving IC50 and R^2 comparable to standard assays. Importantly, by using SemaCytes assay time was reduced by 20% and removed the need for the continual culture of cell lines which require time and material resources and may compromise assay reproducibility.