SemaCyte® Seeding Dish 20 Multiplex (SD20-MX)

Protocol to generate adherent assay-ready cells on SemaCyte® microcarriers, pool barcoded cells, and perform multi-day drug assays with immunostaining endpoints.

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Introduction

SemaCyte® assaying microcarriers function as ultra-miniaturized wells suspended in liquid. SemaCytes loaded with adherent cells can be frozen and thawed while maintaining cell morphology and cell adhesion during freezing. They can be dispensed with manual or robotic liquid handling tools into multi-well plates and are compatible with plate reader and microscopy-based cell assays. SemaCyte® microcarriers with unique barcodes can be pooled and instantly dispensed onto a drug library, enabling identification of different cell types within a well of a microplate during image analysis.

This protocol outlines each of the steps that need to be performed for the preparation of adherent cell-loaded SemaCyte® microcarriers and their use for cell multiplexing during drug incubation assays with immunostaining endpoints.

If the freezing of cells on SemaCyte® microcarriers is required, refer to the following protocol: "SemaCyte® Seeding Dish 20 (SD20) - Basic protocol to generate adherent assay-ready cells on SemaCyte® microcarriers."

The SemaCyte® Multiplexing Workflow

Screening a variety of different cell models can be an operational bottleneck. With the SemaCyte® Seeding Dish it is possible to generate barcoded adherent cells that be pooled together for in-well cell multiplexing.

Cells can be seeded on the SemaCyte® Seeding Dish as per standard cell culture protocols. The Seeding Dish contains arrays of immobilised, barcoded SemaCyte® microcarriers functionalised with bio-polymers to facilitate cell attachment and adhesion. Depending on the loading density and cell type used, each standard SemaCyte® can host 4 to 20 cells.

Once the right confluency and morphology is achieved, cell-loaded SemaCytes are released from the Seeding Dish into suspension and purified. Different cell types seeded on SemaCyte® microcarriers with different barcodes can then be pooled together and dispensed into microplates. These plates can contain pre-prepared drug libraries and can be incubated for multi-day drug assays with imaging endpoints.

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Alternatively, cell-loaded SemaCytes can be aliquoted into cryovials for cryopreservation and stored as assay-ready adherent cells. They can be thawed, pooled, and assayed after 1 hour of recovery. This approach drastically reduces assay preparation time and resources spent on maintaining cells in culture. The production of batches of assay-ready cells on SemaCytes also reduces experimental variability and increases reproducibility.

The SemaCyte® Seeding Dish 20 (SD20)

SemaCytes are provided as immobilised arrays in a petri dish, called a SemaCyte® Seeding Dish 20 (SD20). Each dish has a diameter of 60 mm, a total area of 21.5 cm^2, and 50,000 immobilised microcarriers. Each standard SemaCyte® microcarrier has a cell attachment and growth area of 100 µm x 100 µm, or 0.01 mm^2, coated with a fibronectin mimetic polymer. Alternative sizes and coatings are also available. Each SemaCyte® is enclosed by a cell-repellent wall and they can accommodate approximately 4-20 cells, depending on the cell type. Barcoded SemaCytes have unique optically visible barcodes incorporated into their walls that allow for identification of different populations of cells in pooled SemaCyte samples using Semalyse software.

The recommended density of SemaCytes per well ranges from 40-200 for 96-well microplates and 10-50 for 384-well microplates. Thus, each SD20 contains enough microcarriers for 2-10 microplates worth of experiments.

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SemaCyte® Starter Kit

The Seeding Dish 20 (SD20): 50,000 arrayed microcarriers in a 60 mm dish

The SemaPure15: purifies SemaCytes after cell attachment

The SemaPlate: orientates and immobilises SemaCytes inside microplates

Required Materials & Reagents

- SD20 (SemaCyte® Seeding Dish 20)
- SemaPure15 (SemaCyte® Purification Magnet)
- SemaPlate (SemaCyte® Dispensing Magnet)
- Complete medium (cell type dependent)
- Detaching solution (TrypLE™, trypsin, Accutase® etc.)
- Phosphate-buffered saline (PBS)
- Drug compound(s) of choice
- Fixative solution (paraformaldehyde, formalin, methanol/acetone etc.)
- Permeabilising solution (Triton X-100, Tween-20, methanol/acetone etc.)
- Blocking solution (bovine serum albumin/foetal bovine serum in PBS etc.)
- Primary and secondary antibodies
- Multi-well plates
- Multi-channel pipette
- Wide bore pipette tips

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Protocol Overview

The SemaCyte® multiplexing protocol can be broken down into 6 steps as shown in **Table 1** and **Figure 1** and integrates seamlessly with routine culturing procedures and protocols. A suspension of the cell types of interest is prepared and seeded onto SemaCyte® Seeding Dishes (Day 0) with different barcodes, one for each cell type. After incubation for 18-24 hours or more, depending on the cell type, SemaCytes are collected, purified, pooled, and plated onto drug compound libraries in multi-well plates (Day 1). On the final day of the assay, the SemaCytes are fixed and then sequentially labelled with primary antibodies for the marker of interest and suitable secondary antibodies before imaging.

	Steps	Timing
Day 0	A. Cell preparation B. Cell seeding on the SD20	Dependent on cell type, approximately 15 min
Day 1	C. Preparation of the drug compound library in multi-well plates	30 min
	D. Collection and purification of cell-loaded SemaCytes	45 min
	E. Pooling and dispensing of cell-loaded SemaCytes onto drug libraries	30 min
Final day of assay	F. Fixing, immunostaining and imaging SemaCytes	1-2 days

Table 1 *SD20 cell seeding timeline summary.*

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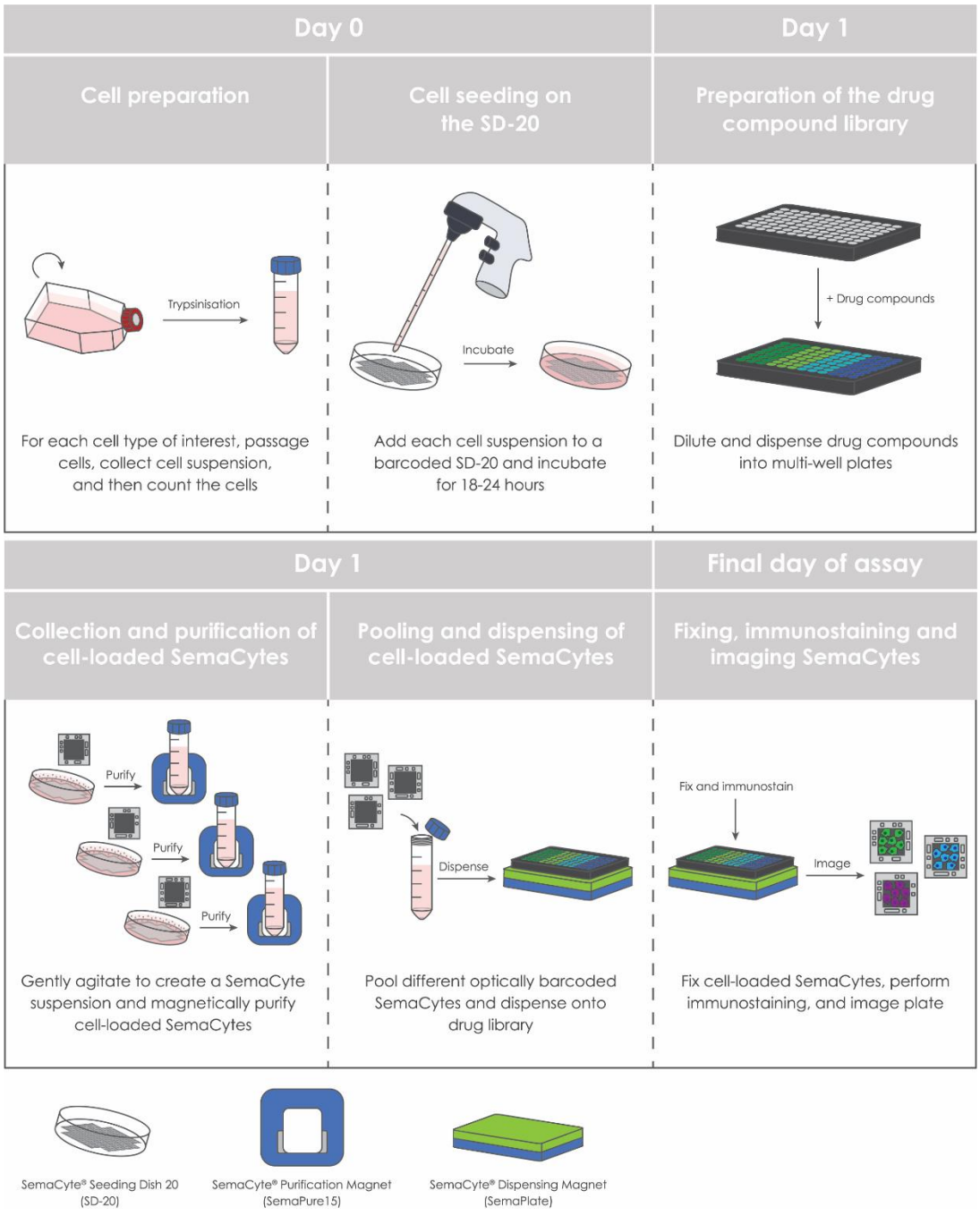

Figure 1 *SD20 cell seeding protocol.*

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Protocol for the generation of adherent assay-ready cells on SemaCyte® microcarriers

A. Cell preparation

1. Passage and culture the cell type of interest until cells are ready to be loaded onto SemaCyte® assaying microcarriers.
2. Aspirate cell medium.
3. Wash cells with Mg^{2+}/Ca^{2+} -free PBS.
4. Add a cell detachment solution to detach adherent cells according to the culturing protocol for the cells of interest. [a]
5. Collect cells in complete medium.
6. Centrifuge cells at an appropriate speed for the cell type used (100-300 × g for 5 min is usually fine for most cells).
7. Remove supernatant.
8. Resuspend cells in complete medium. [b]
9. Count cells.

a) Cell preparation may vary slightly depending on the cell type used. Always make sure to obtain a single cell suspension before seeding cells on SemaCytes.
b) Cells that are prone to clumping can be pre-filtered using cell strainers before seeding.

B. Cell seeding on the SD20

1. Unpack the SD20 and place on the 'cells up' side of the SemaPlate.
2. Dilute enough cells in 5 mL (for 60 mm dish with surface area of 21.5 cm^2) to obtain the desired seeding density. [a]
3. Slowly dispense the cell suspension onto the SD20. **DO NOT** swirl the liquid after adding the cells, doing so may cause the SemaCytes to release and move. [b]
4. Place the SD20 into the incubator for 18-24 h or until the cells have attached.

a) The seeding density required to obtain a sub-confluent or confluent layer of cells on SemaCytes may vary between cell type, assay duration and format. As a

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starting point, seeding density for cells on SemaCytes should be similar to that of the same assay on standard plates.

b) Some cells may benefit from leaving the dish in the hood for 1 h before moving it to the incubator. This could facilitate attachment due to less vibration.

C. Preparation of the drug compound library in multi-well plates

1. Prepare the compound library, including dilutions and replicates, such that each well contains double the desired concentration in half of the final volume. [a]

a) Cell-loaded, pooled SemaCytes will be added onto the drug library at a 1:1 ratio. Ensure that the desired end-concentration of both the drug compounds and SemaCytes are achieved. In this example, 50 µL or 25 µL of the drug library would be prepared in 96-well or 386-well, respectively. In the Step E, 50 µL or 25 µL of SemaCytes will then be added for a final volume of 100 µL or 50 µL. Alternative volumetric ratios are possible.

D. Collection and purification of cell-loaded SemaCytes

1. Using a 1,000 µL pipette, gently aspirate media and re-dispense it over the SemaCytes to dislodge them. This process of pipetting up-and-down can be used multiple times to ensure all SemaCytes are liberated from the surface. Alternatively, SemaCytes can be dislodged by shaking or swirling the dish.
2. Collect the suspension of SemaCytes and gently add it to a 15 mL tube. [a]
3. Gently invert the tube to ensure all SemaCytes are in suspension and then place it into the SemaPure15 purification magnet. [b]
4. Wait for 5 min until all the SemaCytes have been captured by the SemaPure15. **DO NOT** twist or turn the tube within the SemaPure15. [c]
5. Gently remove medium from the top of the tube until 1 mL is remaining. [c]
6. Carefully remove the tube from the SemaPure15. **DO NOT** twist or turn the tube when removing from the SemaPure15. [d]
7. Add 3 mL of fresh medium, invert the tube to resuspend the SemaCytes, and place the tube back into the SemaPure15.

8. Wait for 1-2 min until all SemaCytes have been fully captured by the SemaPure15. **DO NOT** twist or turn the tube within the SemaPure15. [c]
9. Gently remove 3 mL of medium from the bottom of the tube. [c]
10. Repeat steps 7-9 once (or twice) more.
11. Carefully remove the tube containing the cell-loaded SemaCytes in 1 mL medium from the SemaPure15. **DO NOT** twist or turn the tube when removing from the SemaPure15. [d]

a) If there are still SemaCytes in the dish, an additional wash with 1-2 mL of fresh medium can be performed to collect the remaining SemaCytes.
b) The SemaPure15 is used to remove debris and unattached cells to obtain a clean solution of SemaCytes loaded with cells.
c) Do not move, twist, or turn the tube during the purification process. Take care not to touch the SemaCyte pellet with the pipette. Make sure that the liquid level does not drop below the magnet.
d) Do not twist or turn the tube while removing it from the SemaPure15. Pull the tube out with a straight vertical motion.

E. Pooling and dispensing of cell-loaded SemaCytes onto drug libraries

1. SemaCytes with different barcodes and different cell types collected and purified in Step D can now be pooled together. Invert the tubes to resuspend the SemaCytes and mix equal volumes of each in a fresh 15 mL falcon tube to produce a pooled population of uniquely barcoded SemaCytes. [a]
2. Dilute the pooled SemaCytes in complete medium for the desired number of SemaCytes per well. [b]
3. Place the microplate required for the assay of choice onto the SemaPlate, ensuring that the "cells up" side faces up. [c]
4. Dispense the SemaCytes into wells. [d]
5. Remove the well plate from the SemaPlate. **DO NOT** shake or swirl the well plate again.
6. Incubate the plate for the desired duration.

a) Following the final purification in Step D, each tube will contain SemaCytes in 1 mL of medium. Fresh cell medium can be added to each tube prior to mixing equal volumes. When SemaCytes are more diluted, less shear stress will be observed during pipetting.
b) The suggested number of SemaCytes per well is 40-200 for 96-well microplates and 10-50 for 384-well microplates. The SemaCytes will be dispensed directly onto the drug library. In case of the 1:1 ratio described instep C, 50 µL or 25 µL of the SemaCyte® solution would be dispensed in 96-well or 386-well, respectively.
c) The "cells up" side of the SemaPlate ensures that the SemaCytes are pulled down and the cells are dispensed facing up.
d) If using a multi-channel pipette for dispensing, move the dispensing reservoir laterally 5 times between each aspiration. This will ensure homogenous distribution of SemaCytes across the plate.

F. Fixing, immunostaining, and imaging SemaCytes

1. Perform the fixation and immunostaining protocol of choice. [a.]
2. Place the stained microplate onto the SemaPlate, ensuring that the "cells down" side faces up and wait for 10-15min. [b]
3. Image the microplate.
4. Analyse the images with Semalyse and/or other image analysis tools.

a) When exchanging liquids, place the microplate onto the SemaPlate, ensuring that the "cells up" side faces up ensures that the SemaCytes are pulled down. Aspirate ~90% of the liquids slowly from the side to unsure that no SemaCytes are removed during the wash steps. Gently add liquids – still on the SemaPlate – to ensure that the SemaCytes are not pushed to on side of the well.
b) The "cells down" side of the SemaPlate ensures that the SemaCytes flipped over so that the cells are facing down. This ensures the best imaging quality for inverted fluorescent microscopes. In case not all SemaCytes flipped over or in case they have been pushed to the side of the well during assaying, the SemaCytes can be redispersed with pipetting up-and-down.

G. Additional suggestions

1. The amount of SemaCytes/cm^2 for a specific assay may depend on the type of endpoint and the incubation time. For example, CellTiter-Glo™ assays 24-72 hours after dispensing can be performed with as little as 90 SemaCytes/cm^2.
2. The exact concentration of SemaCytes can be determined by adding a 10-100 µL sample of SemaCytes into a multi-well plate and manually counting the number of SemaCytes.
3. The cell health, morphology, and confluency can be assessed after SemaCyte collection and thawing by adding a 10-100 µL sample of cell-loaded SemaCyte® microcarriers into a multi-well plate. While cells can be observed in a brightfield microscope, we recommend using a live/dead staining kit (e.g. AM-Calcein and Ethidium homodimer-1) with a nuclear dye to assess cell viability and recovery using a fluorescence microscope.

For additional information and advice, please contact info@semarion.com. We provide guidance for specific assays or tools.

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