

Protocol

Alvetex™ Strata: Quick Start

1. Alvetex Strata Formats

Alvetex Strata is currently available in the following formats; 12 well inserts ([STP005](#)) and 6 well inserts ([STP004](#)), suitable for short or long term experiments, as well as co-culture and air/ liquid interface set-ups. Inserts are compatible with standard tissue culture plates and with REPROCELL's well insert holder in a deep Petri dish (AVP015), designed for demanding cell types to provide increased volumes of culture media. 12 well inserts fit into both 6 and 12 well plates (to fit into 12 well plates the extended arms must be snapped off). When deciding which Alvetex Strata format to use, the following factors should be considered in combination:

- Cell type and duration of experiment;
- The desired depth of cell penetration into or height of cell multilayer on top of the 3D substrate;
- The type of assay or end point analysis to be performed.

2. Notes Before Starting

- Alvetex Strata is supplied sterile by gamma-irradiation and remains sterile until opened.
- Always handle Alvetex Strata with care, using gloves. Flat-ended forceps are recommended.
- Prior to use, Alvetex Strata must be rendered hydrophilic with a 70 % ethanol wash; add enough ethanol to completely cover the disc, remove, and wash 2× with appropriate culture media. To avoid drying, leave the disc in the final medium wash until required.

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3. Seeding Cells on Alvetex Strata

For optimal results, cells should be seeded dropwise evenly over the entire surface of the scaffold in appropriate media. As a number of cell types prefer to multilayer on top of Alvetex Strata rather than penetrate into it, media should be added below the insert to avoid cells drying out during the initial attachment period and above the insert to encourage even seeding over the entire surface of the scaffold. See Table 1, Figure 2 and Table 2 for specific details for each format.

In brief, when inoculating, aspirate off the wash medium thoroughly from the plate, add medium to the culture well to below and above the insert separately and carefully dispense cells dropwise all over the disc. Replace the lid and incubate at room temperature for at least 30 min to facilitate cell attachment, then transfer to a humidified incubator at 37 °C with 5 % CO₂ overnight to complete cell attachment.

After this time, gently flood the wells with medium to the desired level, see Figure 2 and Table 2 for specific details for each format. With 3D cell culture there are likely to be many more cells growing per unit volume of medium, therefore media changes may be required more frequently than with 2D cultures.

Alvetex Strata format	Recommended cell seeding density*	Recommended cell seeding volume	Initial incubation period
6 well insert in 6 well plate	0.5–2.0 × 10 ⁶	50–200 µL	30–60 minutes
12 well insert in 6 well plate	0.25–1.0 × 10 ⁶	25–100 µL	30–60 minutes
12 well insert in 12 well plate	0.25–1.0 × 10 ⁶	25–100 µL	30–60 minutes

* Optimal cell seeding density will be cell type specific.

Table 1. Overview of the recommended cell seeding densities and volumes for the different Alvetex Strata formats.

4. Monitoring Cell Attachment and Growth via Light Microscopy

Cell culture in Alvetex Strata allows the formation of multilayered, high-density cell populations which approximate the complexity and structure of *in vivo* tissues. When viewing an unstained, unsectioned Alvetex Strata 3D culture under a standard brightfield microscope, the combined density and thickness of the substrate and the 3D culture within it prevent the clear visualisation of individual cells. However, to overcome this, common visible dyes such as Neutral Red staining solution can be successfully used to confirm cell attachment or to check confluency (Figure 1).

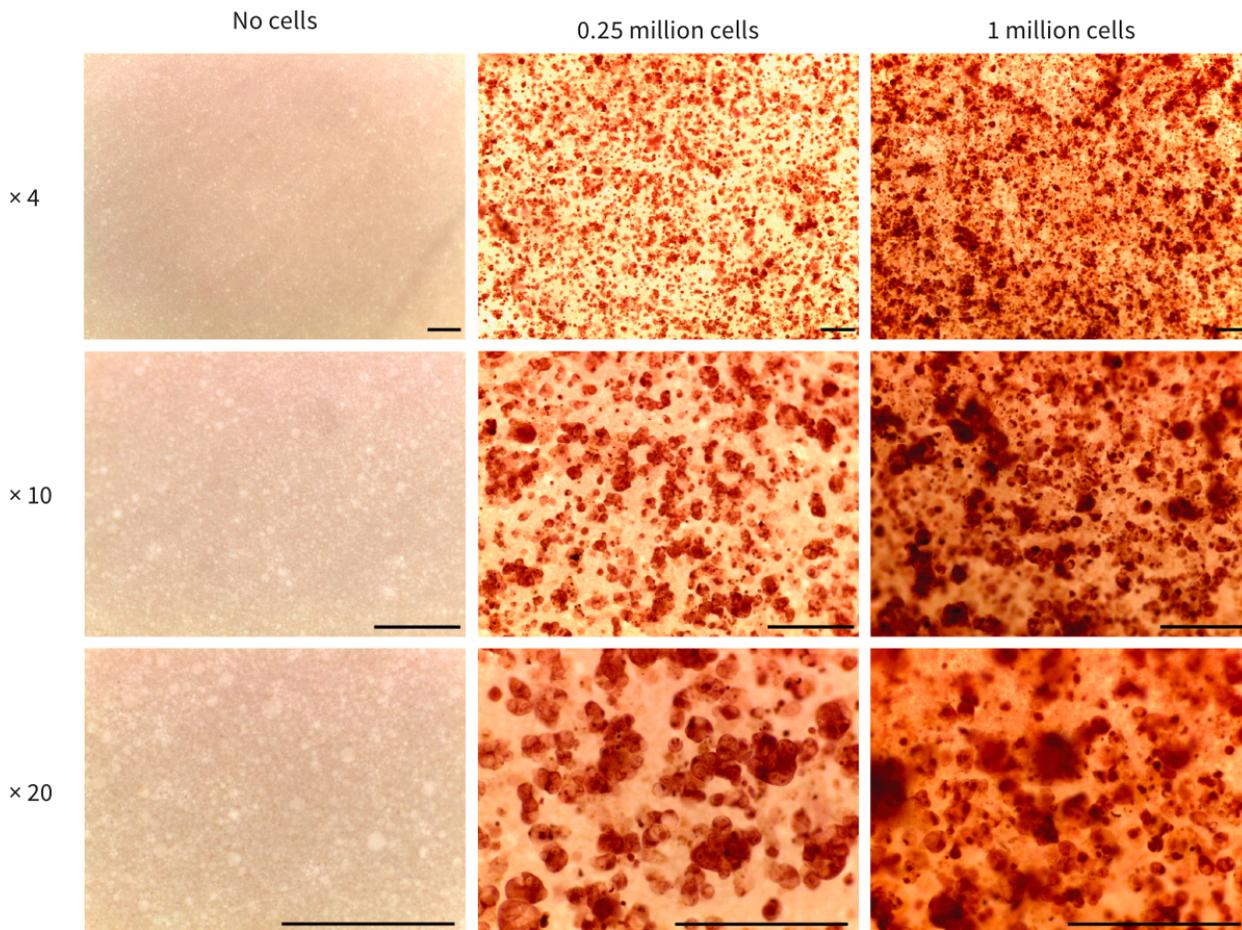
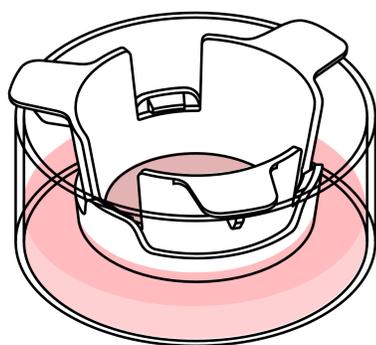
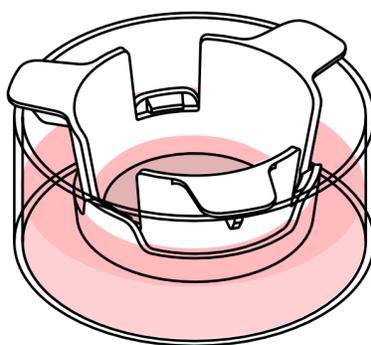


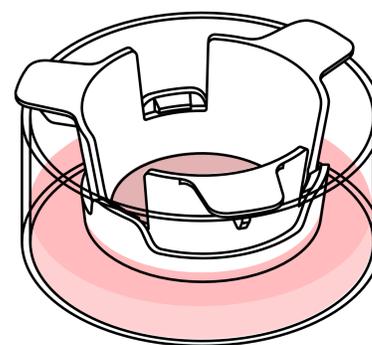
Figure 1. Microscopic appearance of CaCo2 cells (a human colorectal adenocarcinoma cell line) seeded on Alvetex Strata 12 well inserts (STP005) in 12 well plates after 24 hours as visualised by Neutral Red staining (see protocol [Simple visualisation of cells on Alvetex Scaffold using light microscopy \(Neutral Red-Methyl Blue dye\)](#)). Scale bars 100 μ m. Note the increase in staining intensity with higher cell numbers.



(i.) Media from below only
for cells grown in 3D at air-liquid interface.



(ii.) Media from above and below
for routine 3D growth of cells with lower-average metabolic activity/proliferation rate OR for experiments where cells are incubated with test substrate in top chamber only for permeability investigations.



(iii.) Media interconnected
for routine 3D growth of cells with high metabolic activity/ proliferation rate.

Figure 2. Options for media filling levels and well insert configurations.

[A.] Using well inserts or plate formats	Media volumes for different feeding options		
	(i) Media from below only	(ii) Media from above and below	(iii) Media interconnected
6 well insert (STP004) in a 6 well plate	3.5 ± 0.5 mL/well	7 ± 1 mL/well	10 ± 0.5 mL/well
12 well insert (STP005) in a 6 well plate	3.5 ± 0.5 mL/well	7 ± 1 mL/well	10 ± 0.5 mL/well
12 well insert (STP005) in a 12 well plate	1.6 ± 0.2 mL/well	2.4 ± 0.2 mL/well	14 ± 0.2 mL/well

[B.] Using 6 well insert (STP004) with well insert holder (AVP015)	Feeding volume		
	(i) Media from below only	(ii) Media from above and below	(iii) Media interconnected
Low	20 mL ± 1 mL	40 mL ± 3 mL	70 mL ± 5 mL
Medium	34 mL ± 2 mL	50 mL ± 3 mL	80 mL ± 3 mL
High	48 mL ± 2 mL	70 mL ± 5 mL	92 mL ± 3 mL

[C.] Using 12 well insert (STP005) with well insert holder (AVP015)	Feeding volume		
	(i) Media from below only	(ii) Media from above and below	(iii) Media interconnected
Low	20 mL ± 1 mL	40 mL ± 3 mL	70 mL ± 5 mL
Medium	34 mL ± 2 mL	52 mL ± 3 mL	82 mL ± 3 mL
High	48 mL ± 2 mL	70 mL ± 5 mL	92 mL ± 3 mL

Table 2 [A-C]. Feeding options for different Alvetex Strata formats.

5. Different cell types show alternative 3D cell growth patterns on Alvetex Strata 6 well insert format (STP004)

Alvetex Strata in the 6 well insert format (STP004) was prepared in a 6 well plate as described above. HepG2 cells (a human hepatocarcinoma cell line) and 3T3 cells (a mouse fibroblast cell line) were plated at a density of 1×10^6 cells per insert. Plates were maintained for 4 days. After preserving in Bouin's fixative the discs were paraffin embedded, sectioned ($10 \mu\text{m}$) and counter-stained following the haematoxylin and eosin protocol available at reinnervate.com. HepG2 cultures predominantly multilayered on top of the matrix, while 3T3 cells invaded into the whole thickness of the matrix. (Figure 3).

It is important to note that the same number of cells were cultured on Alvetex Strata, for the same growth period, yet very different growth patterns resulted with these two cell types.

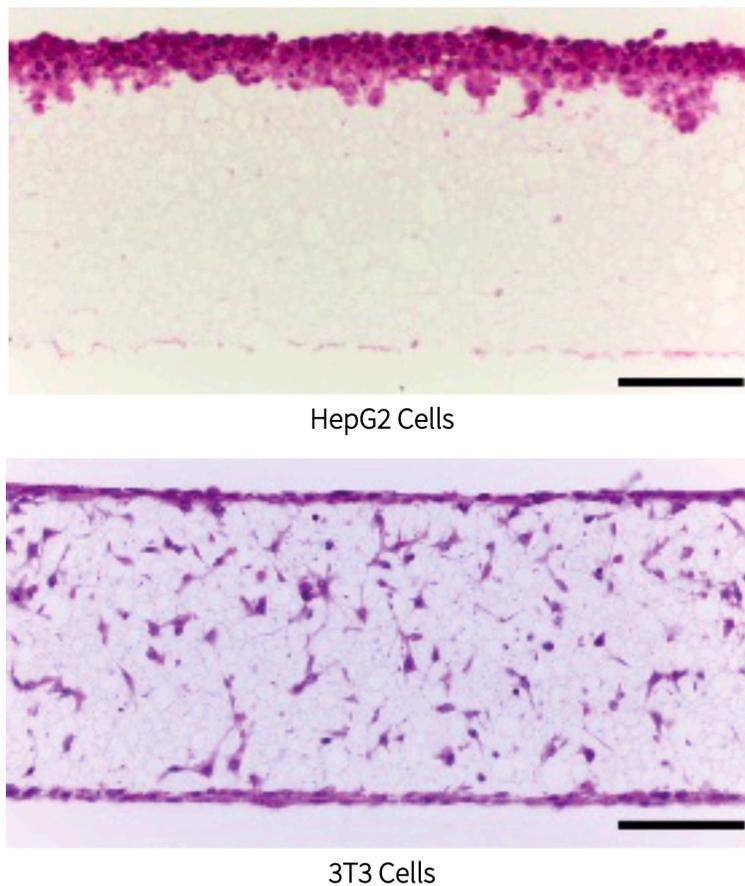
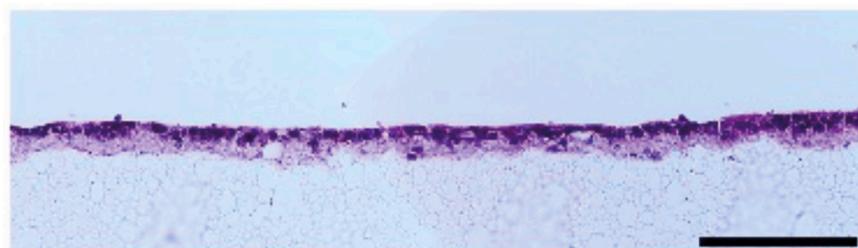


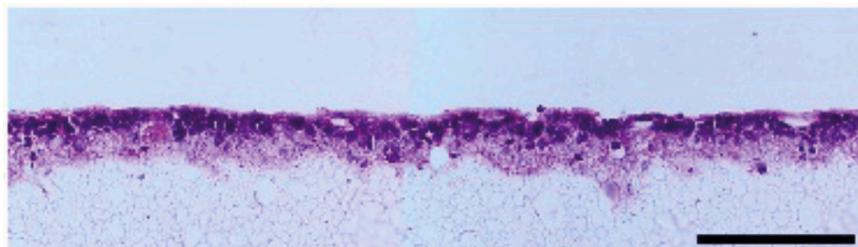
Figure 3. Comparison of the 3D cell growth patterns of HepG2 and 3T3 cells on Alvetex Strata 6 well insert format (STP004). Scale bars $100 \mu\text{m}$.

6. Comparison of the 3D cell growth characteristics of cells cultured on Alvetex Strata presented in various formats

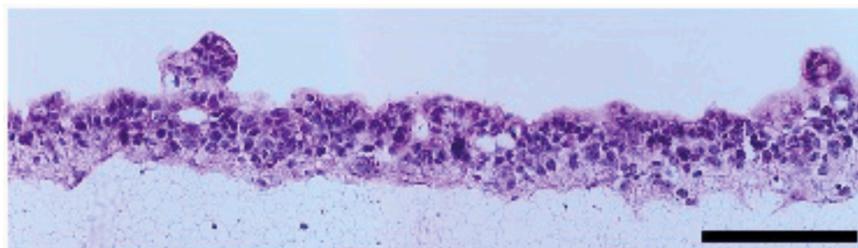
CaCo2 cells were seeded (0.5×10^6 cells per well) on Alvetex Strata in the following formats: 12 well inserts (STP005) in 12 well plate, 12 well inserts (STP005) in 6 well plate and 12 well inserts (STP005) in well insert holder in deep Petri dish (AVP015). Cultures were maintained for 7 days. After preserving in Bouin's fixative, the discs were paraffin embedded, sectioned ($10 \mu\text{m}$) and counterstained with Haematoxylin and Eosin.



12 well insert (STP005) in a 12-well plate



12 well insert (STP005) in a 6-well plate



12 well insert (STP005) in well insert holder in deep Petri dish (AVP015)

Figure 4. Comparison of 3D cell growth patterns of CaCo2 cells grown on various Alvetex Strata formats. Scale bars $100 \mu\text{m}$.

The way in which Alvetex Strata is presented can significantly influence the growth characteristics of the cell culture. Here we demonstrate how CaCo2 3D culture is influenced by the vessel and media volume. Note that the same number of cells were cultured on Alvetex Strata, for the same growth period, yet significantly more proliferation and multilayering resulted on top of Alvetex Strata as the well-size and the medium volume increased, due to greater nutrient availability from above and below the insert. In the case of well inserts contained in a well insert holder in a deep Petri dish, this resulted in the formation of a slab of tissue-like material. During the culture period, media was interconnected as detailed in Figure 2 above and changed fully every 2-3 days, in volumes of 4 mL per well of a 12 well plate, 10 mL per well of a 6 well plate and 75 mL per deep Petri dish.