AgarSqueezer

TROUBLESHOOTING GUIDE

Issue	Comments & suggestions
The agarose gel rotates inside the agar holder	Make sure the agar holder surface is **completely dry and free from any dust, debris, or other contaminants that could interfere with adhesion**. We recommend cleaning with ethanol 70% and distilled water and drying with a blow gun. Ensure that the agarose gel is **thoroughly mixed** and that **no air bubbles** are present before pouring. Always **keep the gel undisturbed and minimize temperature fluctuations** while it solidifies as temperature changes can cause the gel to contract or expand, potentially leading to detachment from the agar holder surface.
Fixed cells are getting detached while removing the agar holder	Fixed cell detachment might be favored by the mechanical stress applied to confined cells, and/or the agar holder removal. There are several things you can implement to maximize cell adherence to their substrate throughout immunostaining procedures: **Coating your substrate** with an adhesive protein or polymer before seeding your cells (the most efficient being Matrigel, alternatively fibronectin/collagen etc) to maximize cell attachment to the substrate in the first place. **Avoid over-confluency**: cells will be more sensitive to mechanical stress and tend to detach more easily from their substrate if they are too crowded. **Reduce incubation time with PFA** (20min at room temperature should be sufficient to fix the cells). **Minimize the number of washing steps** by unmounting the agar holder right after fixation and prior to the washing steps with 3% BSA in PBS. In that case, one incubation with 3% BSA for 20 min should be sufficient.

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Non-adherent cells/3D cellular structures are moving towards the borders when starting confinement	Using a **minimum volume** of culture media when seeding cells will help minimize non-adherent or 3D cellular structure movement when initiating confinement. Add further culture medium once confinement conditions are set.
I am experiencing inconsistent compression after repeated uses	Several parameters can cause incomplete or heterogeneous compression of cells. - **Remaining dirt or agarose residues on the wafer**: usually, using distilled water and cleanroom paper before molding the pillars should allow you to get rid of debris. If not sufficient, you can either gently scrub with a brush, use an airgun to blow away particles or apply a layer of warm liquid agarose and let it dry to peel off all residues. - **Incomplete molding of the pillars**: to avoid the formation of bubbles in the agarose and get a homogeneous agarose layer, maintain the agar holder as vertical as possible upon its contact with the liquid agarose and press evenly while the gel is jellifying. Wait until the gel is completely jellified before moving the agar holder. - **Improper positioning of the agar holder**: make sure the agar holder is properly placed within the device and that nanopillars are in contact with the coverslip.
Not all cells get confined due to the holes in the agarose. How do you take this into consideration when performing molecular analysis?	Cells located under open holes, representing 9.4% of the total population, are not confined under the agarose. While these cells can be easily discriminated when doing live imaging, some tricks can be implemented to reduce potential biases in data analysis when collecting all cells for downstream analysis (qPCR, omics, etc). - First of all, **including appropriate controls** will enable you to normalize your data and therefore highlight the effects of the confinement itself (i.e. confining cells with Agarsqueer using 5 µm or 2.5 µm pillars for the confined conditions, and 30 µm for non-confined control conditions).

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	Micro-patterning methods can be used to **restrict cells to the confined zones**. Check out our **Stencell** products, especially the Quartet and Nonet designs, for an easy method to control cell adhesion and create well-controlled, cell-free areas.
	It is possible to **reduce the number of holes** drilled in the agarose. Additional validation tests will be needed to check for proper cell viability and extend incubation times accordingly when adding external compounds (drugs, fluorescent molecules, etc) to compensate for the reduced speed of diffusion. Always try to maintain an even distribution of open compartments over the cell layer.