Obatala Sciences' Protocol 305
How Do I Create ObaGel® Cultures for *In Vivo* Implantation?

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Reagents, Materials, and Equipment

- Obatala Sciences' ObaGel® (Catalog #OS-301)
- Obatala Sciences' ObaVate™ (Catalog #OS-302)
- Obatala Sciences' Human Adipose-Derived Stromal/Stem Cells (Catalog #OS-101), Human Stromal Vascular Fraction Cells (Catalog # OS-107-01), or equivalent cryopreserved primary cell product
- Obatala Sciences' StromaQual™ Stromal Medium (Catalog #OS-001) or medium of choice
- AdipoQual3D™ Adipogenic Differentiation Medium (Catalog #OS-310)
- Viability stains such as trypan blue or acridine orange/propidium iodide
- 70% ethanol
- 37°C, 5% CO_2 incubator
- BSL2 Biological Safety Cabinet
- Sterile paper towel or kimwipe
- Conical centrifuge tube
- Micropipette and tips
- Well plate and micropipette for desired format, protocol is optimized for 24-well or 96-well plate
- Wet ice for prolonged handling
- Syringe and needle
- Styrofoam or insulated container

Protocol

Initial Handling of Your Obatala Sciences' Products

1. When you receive the package containing your ObaGel® and ObaVate™ products, they may arrive on wet ice or dry ice depending on the shipping conditions.
    a. Prior to use, thaw the unopened products overnight at 4°C until completely thawed. Do not thaw at room temperature or attempt to warm products at higher temperatures.
    b. Aliquot necessary volumes for immediate use into separate containers to avoid repeated freeze/thaw cycles.

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c. After thawing, ObaGel® can be stored at 4°C for <48 hours prior to use. For longer term storage up to 3 months, store at -20°C. For storage >3 months, store at -80°C (shelf life 1 year).
d. After thawing, ObaVate™ can be stored at 4°C for up to 4 weeks, <3 months at -20°C, and up to 2 years at -80°C.

2. After thawing, you may notice protein precipitant present in ObaGel®. This is normal and does not impact function or quality of the product. In fact, it is beneficial to the formation of 3D constructs!

Note: Do not attempt to spin or otherwise remove the precipitant from the product, as it will clump and aggregate. Unaltered, the precipitant will disperse when pipetting.

Seeding Cells in ObaGel® for 3-Dimensional Cultures

1. Thaw ObaGel® and ObaVate™ overnight at 4°C until they are completely thawed. Do not warm to 37°C. Keep reagents on wet ice while in use.
2. Warm growth medium of choice to 37°C.
3. Prepare a 50 mL conical tube with 4 mL of growth medium per vial of cells.
4. Thaw the cryovial of cells until the moment the ice crystals disappear from the vial.
5. Transfer the cryovial to a BSL2 BSC and add the contents of the cryovial dropwise to the 4 mL of growth medium in the conical tube.
6. Homogenize the cell suspension by pipetting several times.
7. Perform a cell count according to your laboratory's standard operating procedures.
8. Centrifuge the conical tube at 1200 RPM for 5 minutes at room temperature.
9. Aspirate the supernatant from the cell pellet and resuspend the cells in ObaVate™ at 1 million cells per mL.
10. From the 1 million cells per mL solution, calculate the total number of cells needed to create the seeding solution.
11. Calculate the volume of concentrated cell solution corresponding to the total cell number from above.
12. Subtract the volume of concentrated cell solution from the total volume of ObaVate™ needed for the seeding solution.[1]
13. Transfer the volume of concentrated cell solution to a new 50 mL conical tube.
14. Add the remaining volume of ObaVate™ needed for the seeding solution.
15. Pipette the cell solution multiple times to mix well.
16. Using a micropipette, vigorously pipette the ObaGel® prior to use.
17. Add the total volume of ObaGel® needed for the seeding solution to the 50 mL conical tube containing the ObaVate™ cell solution such that the seeding solution contains one-part ObaGel® to three-parts ObaVate™ (1:3 ratio). Pipette several times with a p1000 pipette to mix well.

[1] Obatala recommends accounting for pipetting error by increasing the volume by 10%.

18. Once the components are added, quickly transfer the necessary seeding volume of cell suspension onto the tissue culture plate growth surface. Pipette the solution between replicates to maintain homogeneous suspension.[2] [3]
19. After aliquoting, transfer the plate to a humidified 5% CO_2 incubator at 37°C. Observe after 1 hour for initial signs of gelation, and again after 24 hours to visualize cell growth.

Implantation of 3-Dimensional ObaGel® constructs

1. When transporting ObaGel® constructs to the vivarium, do not place on ice. Wrap plates in aluminum foil or appropriately insulated material to aid in temperature maintenance. Place inside a Styrofoam container.
2. After anesthetizing the mice, prepare for injections.
3. Aspirate ObaGel® constructs from the individual wells into syringes by removing the needle from the syringe first, aspirating the ObaGel® construct, and finally twisting the needle back onto the syringe for injection.
4. Immediately inject the ObaGel® constructs into the dorsal subcutaneous top, bottom, left, and right locations (4 locations, 4 injections per mouse).

Recommended Protocols

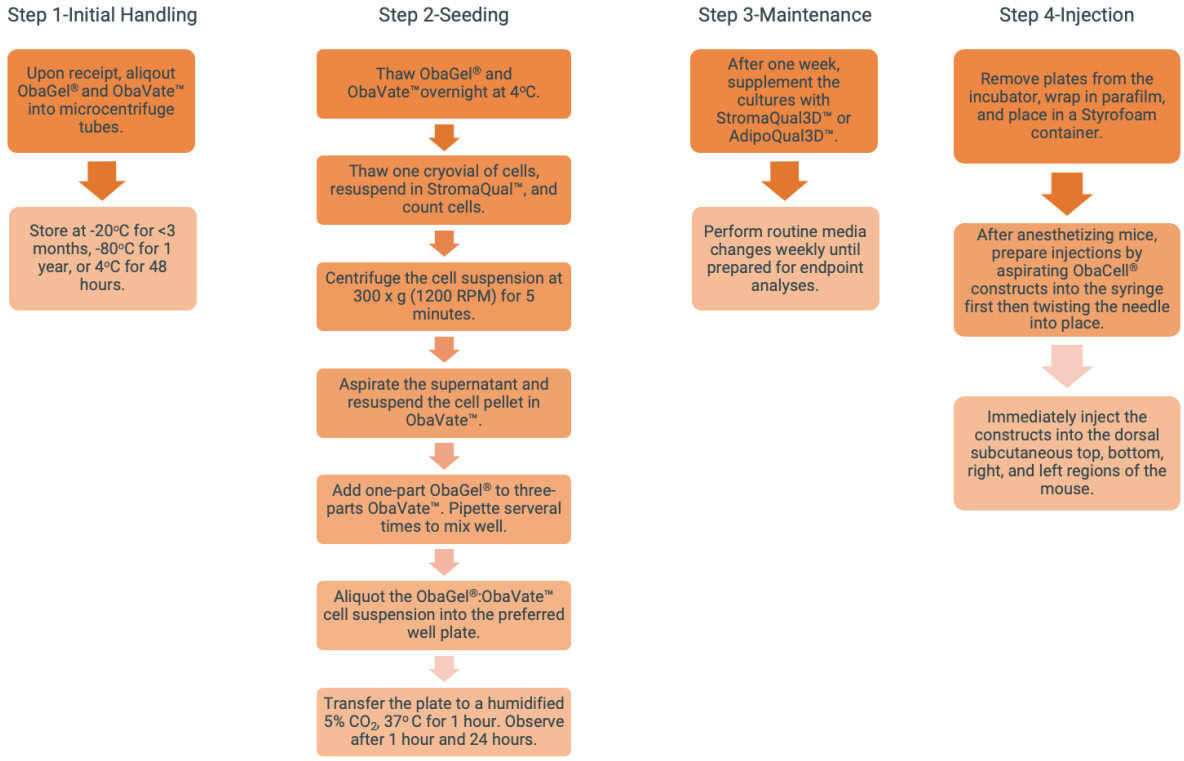
Obatala Sciences' Protocol 101–How Do I Thaw Cryovials of Cells from Obatala Sciences?
Obatala Sciences' Protocol 301–How Do I Create 3D Cultures with ObaGel®?

We expect that you will have new ideas on how to use our product that extend beyond these boundaries and look forward to hearing about novel ways you can use ObaGel® in your discovery research. Please share your findings with us when they become available.

Remember, any laboratory that mentions Obatala Sciences' products by name in a publication is eligible for a 10% discount on their next order! We appreciate not only your business but your endorsement of our products!

[2] Keep suspension on ice while in use.
[3] See Appendix C for recommended seeding volumes.

Appendix A: Establishment of ObaCell® Cultures for In Vivo Implantation Workflow



Appendix B: Troubleshooting

Problem	Reason	Solution
No gelation observed	ObaGel® pre-warmed at 37°C or equivalent prior to use	Do not heat ObaGel® prior to use, maintain on ice during use.
	Repeated freeze-thaws	Aliquot ObaGel® into smaller volumes and thaw as needed.
	Improper extended storage	Store at –20°C for up to one year from receipt date. Thaw and store at 4°C for no more than 48 hrs prior to use.
Gelation during pipetting or handling	ObaGel® pre-warmed at 37°C or equivalent prior to use	Do not heat ObaGel® prior to use, maintain on ice during use.
	ObaGel® not maintained at 4°C or on ice prior to use	Ensure ObaGel® is kept at 4°C or on wet ice prior to use.
Contraction of 3D constructs	Physical disruption of the gel	Careful handling of the plates and manipulation of the constructs. Slowly remove and add media from edge of the well, tilt the plate to reduce shear stress at the gel-liquid interface.
	Inhomogeneous seeding solution	Pipet ObaGel®/ObaVate™ cell suspension vigorously immediately prior to seeding until solution appears homogenous. Pipette solution while seeding, every 3-6 replicates.
Rapid spheroid or organoid formation in 3D constructs	Too much media	Reduce media volume.
	Initial seeding density of cells is too high	Reduce seeding density.
	Inhomogeneous seeding solution	Pipet several times to mix well and pipette several times between replicates.

Appendix C: Recommended Volumes

Reagent	6-well (5 mL)	24-well (1 mL)	96-well (100 µL)
ObaGel®	1.25 mL/well	250 µL/well	25 µL/well
ObaVate™	3.75 mL/well	750 µL/well	75 µL/well
Total Construct Volume	5 mL/well	1 mL/well	100 µL/well
hSVF cells	5,000,000 cells/well	1,000,000 cells/well	100,000 cells/well
hASC cells	500,000 cells/well	50,000-100,000 cells/well	5,000-10,000 cells/well
3D Medium Feedings	2.5 mL/well	500 µL/well	50 µL/well