Protocol graciously provided by: Dr. Mariano Viapiano, Viapiano Lab protocols (© 2006-2014)

C. Preparation of aggregates for adherent cells

1. Incubate agar plates at 37 ˚C for 1h with 2 ml of culture medium for the desired cell type. This will hydrate the agar, otherwise the cells will attach to the plate.
2. Remove the medium after 1h (it will remove debris in the agar) and add 1-2 ml fresh culture medium.
3. Trypsinize and count cells. Add 50,000-70,000 cells/agar plate.
4. Rock agar plates gently to evenly disperse the cells. If possible do this every few hours to prevent formation of loose sheets instead of tight aggregates. Fast and continuous rocking (with a cell culture rocker in the incubator) may aggregate all the cells in the center of the agar plate, so it's not recommended (but can be tested for each cell type).
5. Incubate at 37 °C in a CO_2 incubator for at least 24h.
6. To stain the aggregates, add Green CellTracker (Invitrogen C2925, 10 mM stock) at a final dilution 1/2000 for 60 minutes at 37 °C. This is the CellTracker dye that has the least or no effect on cell motility (CellTracker orange and the Vybrant dyes DiI and DiO reduce cell motility!).
7. To remove the excess dye, collect aggregates with a pipette and transfer to a clean 35mm dish with culture medium. Collect them again (gently) and transfer them to a second clean 35mm dish with culture medium.