

Harvesting adherent cells

1. Grow cells in tissue culture flasks or dishes to confluency (typically, a total of 200 million cells is needed per experiment).
2. Aspirate the media from the plates, rinse cells once with room temperature PBS and trypsinize.
3. Stop trypsinization by adding the complete media to the plates.
4. Resuspend the cells into a single-cell suspension and count their concentration (either with an automated cell counter or hemocytometer).
5. Transfer the cells to 50 ml tubes (up to 50 million cells per tube).
6. Pellet the cells at **800 g** for **4 min** at **room temperature** and aspirate the media.
7. Resuspend the cells in ~0.8 ml PBS per million cells (~40 ml per 50 million cells).
8. Pellet the cells at **800 g** for **4 min** at **room temperature** and carefully aspirate as much PBS as possible.
9. Be ready to immediately move to the "Cross-linking" section of this protocol.

Harvesting suspension cells

1. Transfer cells with media to 50 ml conical tubes (typically, a total of 200 million cells is needed per experiment).
2. Pellet the cells at **200 g** for **5 min** at **room temperature** and aspirate the media. Centrifuge the cells again for **15-30 sec** and remove the remaining supernatant with a pipette tip.
3. Resuspend the pellets in 25 ml of ice-cold PBS.
4. Count cell concentration (either with an automated cell counter or hemocytometer).
5. If cell concentration is higher than 1 million per ~0.8 ml PBS adjust the concentration accordingly and make sure to keep up to 50 million cells per 50 ml tube (40 ml of the adjusted cell suspension per tube).
6. Pellet the cells at **200 g** for **5 min** at **room temperature** and aspirate the PBS. Centrifuge the cells again for **1 min** and remove the remaining supernatant with a pipette tip. Keep tubes on ice until.
7. Be ready to immediately move to the "Cross-linking" section of this protocol.

Materials required for cross-linking:

- PBS
- Cross-linking agent. Use either:
 - o ~37% formaldehyde stock solution in ~10-15% methanol (preferably opened not more than 3 months before usage)
 - o ~50% or ~25% glutaraldehyde stock solution in H₂O (preferably opened freshly)
- Glycine solution (typically 2.5 M) in PBS

Before you start:

Make sure that PBS, Glycine solution, and formaldehyde/glutaraldehyde stock solution are all free of contaminations and readily available in sufficient amounts for the planned procedure. Equilibrate them to room temperature if necessary.

Prepare 1% solution of formaldehyde (be mindful that the stock solution is typically ~37% concentrated) or 1% solution of glutaraldehyde (be mindful that the stock solution is typically ~25% or ~50% concentrated) solution in PBS shortly in advance (preferably as shortly as possible, not longer than 1h before use) to make sure that it is ready to use immediately after the tissues are prepared.

Cross-linking

1. Take cellular pellets freshly harvested into 50 ml tubes (free of any excess liquid) and resuspend the cells in a small volume of 1% formaldehyde until no chunks are visible.
2. Add the remaining formaldehyde solution to the tube to the final amount of 0.9 ml per million cells. Mix by inverting and cross-link for **30 min** on a rotator or in a shaker.



3. Add 1 ml of 1.25 M glycine solution per every 10 ml of formaldehyde solution used for the cross-linking and continue incubating for another **5 min**.
4. Pellet the cells at **2000 g** for **5 min** at **4 °C**.
5. Remove the supernatant from the cells and resuspend them in 20 ml of cold PBS, and pellet them again at **2000 g** for **5 min** at **4°C**.
6. Remove the supernatant and resuspend the pellet in 1ml of cold PBS per 20 mln cells.
7. Transfer the cells to 2 ml Eppendorf tubes, pellet them at **2000 g** for **3 min** at **4 °C** and **remove as much PBS as possible** with a pipette.
8. Use cells directly for the following steps of the protocol or flash-freeze the pellets in liquid nitrogen and store them at -80°C.

