Procedure

Briefly the procedure is as follows: Components of the dissociation medium are reconstituted as described previously; minced tissue is added and the mixture is equilibrated with O_2:CO_2. Tissue is dissociated by incubation with activated papain at 37°C, followed by trituration. Dissociated cells are pelleted then resuspended in medium containing ovomucoid, a papain inhibitor. Intact cells are separated from cell membranes by centrifugation through a single step discontinuous density gradient and the pellet finally resuspended in medium appropriate for cell culture or flow cytometric analysis.

For those unfamiliar with tissue dissociation and cell culture techniques, two operations deserve additional explanation.

1. Equilibration with 95% O_2:5%CO_2

It is important for the survival of the tissue during dissociation that the incubation medium be both well oxygenated and buffered at physiological pH. Both requirements are satisfied when the medium is equilibrated with 95% O_2:5%CO_2 . The Earle's Balanced Salt Solution contains a pH sensitive indicator dye. When it is red or purple in color, the medium is too alkaline. This is likely to be the case when the tissue is placed in the papain solution (Step #4), and reequilibration with O_2:CO_2 is usually necessary prior to incuation at 37°C.

Gas should not be bubbled directly into any solution containing protein. This can result in frothing and denaturation of the protein with loss of biological activity. Gas can be sterilized by passage through a sterile fiber plug such as the cotton plug in a sterile Pasteur or volumetric pipette. While mixing the solution, pass O_2:CO_2 continuously through the space above the liquid until the color indicates pH 7.2-7.4 according to the color chart included in the kit. The Earle's Balanced Salt Solution is pregassed but should be equilibrated with sterile O_2:CO_2 each time the bottle is opened. The reconstituted inhibitor should also be equilibrated with sterile O_2:CO_2 before each use.

2. Trituration (cell dispersion through mild pumping action).

This is a crucial procedure. It serves to break up the tissue fragments following incubation in the dissociation mix. If done too vigorously, cells will be destroyed; too weakly and tissue fragments will be left intact. In the context of neuronal tissue, gentle trituration, using a 10 ml pipette, constitutes filling and emptying the barrel at a rate of about 5 ml per second. Avoid bubbling the cell suspension.

Dissociation Protocol:

(Sterile procedures should be used throughout)

1. Add 32 mls of EBSS (vial 1) to the albumin ovomucoid inhibitor mixture (vial 4) and allow the contents to dissolve while preparing the other components. Mix before using and equilibrate with O_2:CO_2. Reconstitute for the first use, then store and reuse.
2. Add 5 mls of EBSS (vial 1) to a papain vial (vial 2). Place vial 2 in a 37°C water bath for ten minutes or until the papain is completely dissolved and the solution appears clear. If solution appears alkaline (red or purple) equilibrate the solution with 95% O_2:5%CO_2. The solution should be used promptly but can be held at room temperature during the dissection. A separate papain vial is provided for each dissociation. (If desired the papain can be transferred to a centrifuge tube or other container before proceeding.)
3. Add 500 µls of EBSS to a DNase vial (vial 3). Mix gently -- DNase is sensitive to shear denaturation. Add 250 µls of this solution to the vial containing the papain. This preparation contains a final concentration of approximately 20 units/ml papain and 0.005% DNase. Save the balance of the DNase vial to use in step #7. A separate DNase vial is provided for each dissociation..
4. Place tissue in the papain solution. Tissue should be slightly minced or cut into small pieces (this can be done separately or on the side of the tube containing the papain). Displace air in vial with sterile O_2:CO_2. Do not bubble gas through the solution. Immediately cap vial.
5. Incubate the vial containing the tissue at 37°C with constant agitation (a rocker platform is ideal) for 30 min to 1 1/2 hrs. The amount of time must be determined empirically; however, embryonic tissue generally requires less time than postnatal tissue.
6. Triturate the mixture with 10 ml pipette. Allow any pieces of undissociated tissue remaining after trituration to settle to the bottom of the tube. Vigorous trituation of neuronal tissue results in a high yield of cells, most of which are spherical and devoid of processes. Gentle trituration results in more undissociated tissue fragments and a lower yield of cells although many of these now retain their proximal processes.
7. Carefully remove the cloudy cell suspension, place in sterile screwcapped tube and centrifuge at 300g for 5 minutes at room temperature. Be careful to avoid including any pieces of undissociated tissue during this time -- prepare medium to resuspend the pelleted cells.

Mix 2.7 mls EBSS (vial 1) with 300 µls reconstituted albumin-ovomucoid inhibitor solution (vial 4) in a sterile tube. Add 150 µls of DNase solution (vial 3) saved at step #3.

8. Discard supernatant and immediately resuspend cell pellet in DNase dilute albumin-inhibitor solution prepared in step # 7.
9. Prepare discontinuous density gradient. Add 5.0 ml of albumin-inhibitor solution (vial 4) to centrifuge tube, carefully layer cell suspension on top, then centrifuge at 70g for 6 minutes at room temperature. The interface between the two layers of the gradient should be clearly visible although minimal mixing at this boundary does not affect the result. Dissociated cells pellet at the bottom of the tube, membrane fragments remain at the interface.
10. Discard the supernatant and immediately resuspend the pelleted cells in medium for cell culture or for flow cytometric analysis.