CHAPTER 1

Introduction

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This introduction presents an overview of techniques and key considerations for the separation and isolation of cellular components. It illustrates how all subcellular fractionation protocols use the same general principles and strategies. The chapters that follow provide specific isolation schemes for particular cellular organelles and intracellular components.

CHOICE OF STARTING MATERIAL

The first decision in isolating any cellular fraction is "how much?" How much of the cellular organelle or component do you need in the final fraction? Traditionally, animal tissues are used because the large numbers of cells yield significant quantities of the fraction of interest. In particular, the protocols for fractionating rat tissue are well-developed, and several chapters here use rat tissues for isolating the cellular compartment of interest. However, advances in proteomics have allowed the proteome of organelles and complex protein fractions to be identified from just a few micrograms of proteins, so relatively small numbers of cultured cells may be required in those cases. Functional assays may still require a large quantity of final material.

One advantage of using tissue culture cells is that the cells can be readily manipulated (e.g., by RNA interference) before performing subcellular fractionation. A knockout mouse is not always available for every gene of interest nor can be generated within laboratory budgets. When using tissue culture cells, a couple of parameters need to be noted: the length of time the cells have been cultured (if adherent) and the confluency of the cells. These conditions can significantly influence the outcome of the fractionation procedure and are often determined empirically. If a protocol in this manual has provided these conditions, then it would be prudent to follow the protocol.

METHODS TO DISRUPT CELLS

After the material to fractionate has been chosen, the cells need to be disrupted. The disruption method needs to be sufficient to break open the cells but not so disruptive that the organelle or fraction to be isolated becomes overly damaged and/or nonrecoverable. Tissues such as the liver, kidney, and brain are readily homogenized using Potter–Elvehjem Teflon or Dounce homogenizers. But more often than not, tissue culture cells can be difficult to break open. Dounce homogenizers may be used with tissue culture cells, but they tend to be more successful with larger cells. In practice, small cells such as macrophages require high shearing forces to break them open, even if they are swollen before homog-

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enization by placing them in a hypotonic solution. This might not be a problem if you wish to isolate the plasma membrane (Chapter 2) and are not concerned whether your lysosomes are intact, but if you wish to isolate intact lysosomes (Chapter 8), then you need to choose a method that can disrupt the plasma membrane without disrupting the lysosomes.

The methods in this manual generally use shearing forces to disrupt cells, such as those generated by the aforementioned Potter–Elvehjem and Dounce homogenizers. But other shearing-force methods in this manual include passing cells through narrow-gauge needles or using ball-bearing homogenizers. When using needles, the gauge of the needle needs to be chosen so that the cell is disrupted but nuclei can pass through unbroken. Care must be taken to fill the needle and syringe beforehand with the homogenization buffer so that no air remains; any remaining air can give non-reproducible results. A ball-bearing homogenizer works by passing cells through a chamber of a fixed size that contains a ball bearing of a fixed size. The clearance is typically on the order of 6–20 μm. Both needles and ball-bearing homogenizers generate shearing forces that are dependent on the force of the operator using the device, so consistency can very much be operator-dependent. Furthermore, the viscosity of the solution being homogenized can affect the shear forces. Cells resuspended in a large volume will break differently than cells resuspended in a small volume, so try to be consistent.

A gentle and sometimes more reproducible technique for breaking open cells is to use nitrogen cavitation. Nitrogen cavitation places the cells under high pressure, allowing the nitrogen to dissolve into the cells. When the pressure is released, the nitrogen dissolved in the cytosol expands and breaks open the cells.

There is not one hard, fast rule for homogenization, and several homogenization methods may work for any particular cell type. Needles and Dounce homogenizers tend to be the cheapest options and are a good starting point if developing a new protocol. But more specialized equipment and methods, such as a ball-bearing homogenizer or nitrogen cavitation, may be required if the results obtained are unsatisfactory. The majority of the protocols in this manual start at a similar point; there are plenty of examples of how to homogenize cells and tissues, even if your starting material differs slightly from those described. An isotonic buffer (300 mOsm) containing 0.25 M sucrose and supplemented with mono- or divalent cations, protease inhibitors, and chelating agents is routinely used for the homogenization buffer. The isotonic buffer prevents organelles from being disrupted, but if cells are homogenized in a hypotonic buffer to aid homogenization, then isotonicity should be restored as soon as possible.

FRACTIONATION

After homogenization, the cellular components need to be separated/fractionated. Again, the protocols in this manual, for the most part, use the same technique: differential centrifugation. During centrifugation, cellular components are pelleted based on size, density, and sedimentation properties. Several centrifugation steps with increasing centrifugal force can effectively, albeit crudely, fractionate the cell. Numerous protocols have an initial 5–10-min centrifugation step at ~1000*g* that pellets the nuclei, mitochondria, and sheets of plasma membrane. The resulting supernatant is often referred to as the postnuclear/postmitochondrial supernatant. Subsequent centrifugation steps can then be used to isolate lysosomes, peroxisomes, endosomes, Golgi membranes, and so forth. Although differential centrifugation can generate a fraction enriched for a specific cellular component, often this is a crude mix of membranes. Further purification of the fraction of interest may be achieved by using additional rounds of centrifugation with continuous or discontinuous density gradients.

One major advantage of centrifugation is the ability to separate large volumes of crude tissue homogenates into more reasonably sized volumes for further purification. However, some of the centrifugation steps can be time-consuming. Commonly used rotors for centrifugation are fixed-angle, swinging-bucket, near-vertical, and vertical. With vertical rotors, gradient layers reorientate themselves vertically. Isopycnic centrifugation is achieved more quickly than when using a swinging-

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bucket rotor because the cellular particles do not need to travel as far. However, the resolution of separation is not as sharp.

The medium used for density gradient centrifugation is often sucrose. It is cheap and can be used at concentrations ranging from 2% (w/w) to 70% (w/w), thus producing a range of densities (1.006–1.347 g/mL) that enable most subcellular components to be separated. However, at high concentrations, sucrose is hyperosmotic and organelles undergo shrinkage, which can limit the ability of sucrose gradients to resolve subcellular components on the basis of density and size. Furthermore, if organelles are to be used in functional assays, then the lack of isotonicity may be detrimental to the function of the organelle being tested. There are, however, other gradient media that can be used to maintain iso-osmotic conditions over a range of useful densities. These include Percoll, Ficoll, and Nycodenz.

The fractionation procedures very much depend on the level of purity required of the final fraction. If the properties of the organelle are to be studied, then quantity is sacrificed over purity, and there are likely to be numerous centrifugation steps. If simple associations between proteins and organelles are to be examined, then an initial crude separation and a single gradient are often sufficient. The functionality of the organelle is paramount for functional assays, and as long as other membranes will not interfere with the assay, then purity may not be important.

ASSESSING FRACTIONS

Although the protocols in this manual are tried and tested, when using any protocol for the first time, it is advisable to retain fractions from each step so that the effectiveness of the protocol can be assessed. Fractions retained during the procedure can be used in protein assays (e.g., western blotting) or in enzyme assays for particular organelles (e.g., galactosyl transferase, GalT, for Golgi [Chapter 12] or β-hexosaminidase for lysosomes [Chapter 8]). By assessing the fractionation procedure, the investigator can evaluate the yields, fold purifications, purity, and contaminants of the sample at every step. This allows troubleshooting and modifications to the protocol to be undertaken if necessary.

THE PROTOCOLS

The following chapters in this manual provide step-by-step protocols for isolating the following organelles and components: plasma membranes, clathrin-coated vesicles, early and late endosomes, synaptosomes, phagosomes, exosomes, lysosomes, GLUT4 vesicles, peroxisomes, microtubules and microtubule-associated proteins, tubulin, microtubule motors, Golgi stacks, mitochondria, chloroplasts, rough microsomes, ribosomes, nuclei, and the nuclear matrix. Some of the methods are robust and have not been altered significantly since their inception, whereas others make use of the latest technologies to aid in the isolation of cellular components.