Isolation of organelles from protozoa and amoeba

- ♦ OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- ♦ To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- ♦ **For the isolation of acidocalcisomes see Application Sheet S48**

1. *Trypanosoma brucei*

1a Glycosomes

1a-1. Background

Isolation of glycosomes from *Trypansoma brucei* in double sucrose gradients, in Nycodenz® gradients and in Percoll® gradients was compared by Aman and Wang [1]. Those isolated in Percoll® produced the least enriched fraction and while those in Nycodenz® were marginally less homogenous than those from the double sucrose gradient, the convenience of using a single gradient of a more particle-friendly medium may be more important [1-3]. More recently however a more convenient OptiPrep™-based method has been developed by Colasante et al [4].

1a-2. Solutions required

A. OptiPrep™

B. Homogenization medium: 0.25 M sucrose, 1mM EDTA, 0.1% (v/v) ethanol, 5 mM Mops pH 7.2.

C. 6 mM EDTA, 0.6% ethanol, 30 mM Mops, pH 7.2.

D. 1 M sucrose.

E. Gradient solutions: Make up from solutions A, C, D and water using respectively, these ratios by volume:

E1: 5 + 0.6 + 0.4 + 0.0 (50% iodixanol)
E2: 4 + 0.6 + 0.7 + 0.7 (40% iodixanol)
E3: 2 + 0.6 + 1.1 + 2.3 (20% iodixanol)

Keep the following stock solutions at 4°C:

500 mM Mops	10.45 g per 100 ml water
100 mM EDTA ($Na_2 \bullet 2H_2O$)	3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 0.2 ml, 2 ml and 2 ml respectively of ethanol, EDTA stock and Mops stock; adjust to pH 7.2 with 1 M NaOH and make up to 200 ml.

Solution C: Add 0.6 ml, 6 ml and 6 ml respectively of ethanol, EDTA stock and Mops stock to 50 ml water; adjust to pH 7.2 with 1 M NaOH and make up to 100 ml.

Solution D: Dissolve 34.2 g sucrose in 50 ml water and make up to 100 ml.

Add protease inhibitors as required to solutions as required. Solution A can also be used as a wash medium (see Section 2d) for the trypanosomes; protease inhibitors may be omitted from the solution when used for this purpose.

1a-3. Ultracentrifuge rotor requirements

Vertical rotor with a tube capacity of approx 38 ml tubes (e.g. Beckman VTi50 or Sorvall TV860) with appropriate sealed tubes (see Section 2f, Notes 1and 2).

1a-4. Pre-gradient protocols

See Colasante et al [4] for details of harvesting, washing and homogenization of the trypanosomes by grinding with silicon carbide.

1a-5. Centrifugation protocol

Carry out all operations at 0-4°C

1. Remove the silicone carbide by centrifugation at 100 *g* for 15min.
2. Decant the supernatant and centrifuge at 1000 *g* for 15 min to pellet the nuclei.
3. Decant the supernatant and centrifuge at 17,000 *g* for 15 min to give a pellet of crude glycosomes.
4. Decant and discard the supernatant and resuspend the pellet in approx 3 ml of Solution B using a few gentle strokes of the pestle of a loose-fitting Dounce homogenizer (Wheaton Type B).

5. During the differential centrifugation steps use a two chamber gradient maker or a Gradient Master to prepare linear gradients from 16 ml each of gradient solutions E2 and E3 in tubes for the vertical rotor and underlayer each gradient with 3.5 ml of gradient solution E1. Transfer the suspension from step 4 to the top of the gradient and centrifuge at 170,000 *g* for 1 h. Use a slow acceleration and deceleration programme if available; alternatively turn off the brake during deceleration from 2000 rpm (see Section 2f, Notes 3 and 4).

6. Harvest the purified glycosomes, which form the densest band in the gradient or unload the gradient in a number of equal volume fractions. For more information on gradient collection see **Application Sheet S08**.

1a-6. Notes
1. If the preparation is scaled down to smaller tubes (13 ml) use the Beckman VTi65.1 or Sorvall 65V13 rotor. If no vertical rotor is available then a swinging-bucket rotor may be used but the centrifugation time will need to be increased to take account of the longer path length.
2. The sealed tubes that are the easiest to load and unload are Optiseal™ tubes for Beckman rotors.
3. For smaller volume rotors scale all gradient and sample volumes down proportionately. For methods describing the construction of continuous gradients see **Application Sheet S03**.
4. A continuous gradient of 20%–35%, v/v OptiPrep™ (with a 50%, v/v OptiPrep™ cushion) was used by Gualdron-López et al [5], centrifuged at 100,000 *g* for 2 h in a vertical rotor. Later the centrifugation time was extended to 15 h [6].
5. See also Section 3d below.

1b. Contractile vacuole

The supernatant from a series of low-speed centrifugations was centrifuged at 100,000 g for 1 h and the resuspended pellet median-loaded (25% iodixanol) in a discontinuous gradient of 15%, 20%, 25%, 30%, 34%, 37% and 40% (w/v). It was centrifuged at 50,000 g 65 min; the contractile vacuole banded near the top of the gradient [7,8].

1c. Lipid rafts

For the isolation of these structures **see Application Sheet S32 and Mini-review M06**

1d. Acidocalcisomes

For the isolation of these structures **see Application Sheet S48**

2. *Dictyostelium*

2a. Acidocalcisomes

For the isolation of these structures **see Application Sheet S48**

The iodixanol gradient also provides mitochondria- and contractile vacuole-rich fractions from *Dictyostelium*. Sivaramakrishnan and Fountain [9] reported that iodixanol gradients also resolve the ER from contractile vacuoles.

2b. Phagosomes (*Legionella*-containing)

A post-nuclear supernatant from which the lysosomes had been removed by antibody binding, was centrifuged through a 5-30% (w/v) iodixanol at 100,000*g* for 2h [10]. The phagosomes banded approx. a third of the way down the gradient. Denser *Legionella*-containing vacuoles partially overlap the mitochondria; this was interestingly resolved by increasing the density of the mitochondria by addition of iodophenylnitrophenyltetrazolium (INT) to the homogenate [11].

♦ Iodixanol gradients have also been used in the analysis of acyl CoA binding protein (AcbA) which is unusually sequestered into a dense vesicle population in a mutant form of the protozoan [12]

3. *Lieshmania*

3a. Acidocalcisomes

For the isolation of these structures **see Application Sheet S48**

3b. Lipid rafts

For the isolation of these structures see **Application Sheet S32 and Mini-review M06**

3c. Subcellular membranes

A low-speed supernatant was loaded on to discontinuous 18.5%, 28% and 40% (w/v) iodixanol gradients, centrifuged in a Beckman SW41 rotor at approx 190,000 *g* for 90 min [13].

3d. Glycosomes

A method, alternative to that described for *Leishmania* glycosomes (see Section 1) is to use a density barrier [14,15]. The crude glycosome containing fraction was produced at 26,000 *g* for 50 min (rather than the conditions described in Step 7); 2 ml of this was layered over 10 ml of 30% (w/v) Nycodenz® and centrifuged at 105,000 *g* for 50 min to pellet the glycosomes.

4 Hydrogenosomes

4.1 *Mastigamoeba balamuthi*

A discontinuous iodixanol covering the range 15–40% was centrifuged at 100,000 *g* overnight to resolve a hydrogenosome fraction [16].

4.2 *Trichomonas* hydrogenosomes

Crude hydrogenosomes from *Trichomonas vaginalis* (homogenized by sonication in 0.25 M sucrose, 0.5 mM KCl, 10 mM Tris-HCl, pH 7.2 and the usual protease inhibitors), were prepared from a post-nuclear supernatant by centrifugation at 17,000 *g* for 20 min [17]. The gradient solutions were prepared (containing sucrose, EDTA and Tris buffer) in the same manner as described in **OptiPrep Application Sheet S01**. Either continuous (18-36% w/v iodixanol) or a discontinuous over the same concentration range (in 2% steps. The sample was underlayered in 50% (w/v) iodixanol and centrifuged at 200,000 *g* for 2 h. Hydrogenosomes banded at approx. 30% (w/v) iodixanol. In an alternative method published by Kay et al [18] the crude fraction was suspended in 0.25 M sucrose, 20 mM HEPES, 5 mM DTT containing 6% (w/v) iodixanol and the usual protease inhibitors and layered over a 12-24% (w/v) iodixanol gradient centrifuged at 70,000 *g* for 2 h. The banding position of the hydrogenosomes identified by their characteristic light brown colour. More recently a 15,000 *g* pellet was fractionated in a discontinuous 12-30% iodixanol gradient at 200,000 *g* for 12 h [19]

5. *Giardia*

Giardia contains a lysosome-like organelle [20], which like the mammalian lysosomes has relatively low density. When a 100,000 *g* particulate fraction of a post-nuclear supernatant (prepared from a *Giardia* sonicate) is fractionated in a 1.05-1.25 g/ml Nycodenz® gradient, the organelle bands at approx 1.07 g/ml [20] after centrifugation at approx. 150,000 *g* for 2.5 h.

6. *Paramecium*

Parmecium tetraurelia homogenates have been fractionated on a 10 to 30% (w/v) iodixanol gradient at 46,000 *g* for 18 h in SNARE protein distribution studies [21]

7. *Toxoplasma*

Host cells were fractionated to investigate the organelle association of evacuoles: a light mitochondrial fraction of the cells was adjusted to 20% (w/v) iodixanol and a self-generated gradient formed by centrifugation at 180,000 *g* for 4 h in a small volume near-vertical rotor [22]. More recently “plant-like vacuoles” were isolated from toxoplasma tachyzoites in a discontinuous gradient of 15%, 20% (containing a “microsomal fraction”), 25%, 30%, 34% and 38% iodixanol and centrifuged at 50,000 *g* for 1 h. The vacuoles banded close to the top of the gradient [23].

8. References

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