Isolation of vesicular and granular fractions from various sources

- 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 **2020SMemapp** file and select the appropriate S-number.
- This Application Sheet briefly summarizes the current information on the isolation of granules from the following cell/tissue types (1-6) (see **Note** below regarding neural tissue fractions);
- (1) Adrenal chromaffin cells
- (2) Lymphocytic cells
- (3) Neutrophils and HL-60 cells
- (4) Neuromelanin granules
- (5) Parotid acinar cells
- (6) Platelets
- (7) References

Important Note: Because of the diversity of studies on the neural system and the variety of methodologies, **Section 8** contains an additional list of references sorted according to the nature or source of particle(s) under investigation. Papers are listed alphabetically according to first author.

1. Adrenal chromaffin cells

The gradient methodology which was originally published in 2008 [1] described an homogenization medium of 0.3 M sucrose, 1 mM $MgSO_4$, 1mM EDTA and 10 mM HEPES (pH 7.0). The gradient solutions were prepared from a stock solution containing 50% (w/v) iodixanol: OptiPrep™ was diluted 5:6 v/v with 0.3 M sucrose, 6 mM $MgSO_4$, 6mM EDTA and 60 mM HEPES (pH 7.0) [1-3]. Later [4,5] 10 mM KCl was included in the homogenization medium; consequently the OptiPrep™ diluent contained 60 mM KCl and the sucrose concentration reduced to 0.25 M (to maintain isoosmolality). Preceding the gradient a standard differential centrifugation of 1000 *g* for 10 min to remove nuclei, followed by 10,000 *g* for 20 min to pellet the granules (large dense core vesicles) is common to most methods. The major contaminant of the granules is therefore mitochondria; most (but not all) of the lysosomes and other smaller organelles will remain in the supernatant.

Early gradients [1-3] were discontinuous and comprised iodixanol solutions of 8% and 16% [1,2] or 8% and 18% (w/v) iodixanol [3]; centrifugation conditions were 100,000 *g* for 1 h or [1,2] or 10,000 *g* for 10h [3]. More recently continuous gradients of 8-26% (w/v) iodixanol [4,5] centrifuged at 100,000 *g* for 1 h (sample was applied in 5% iodixanol), allowed the granule fraction to reach the bottom of the tube. Similar gradients are described in ref 6 with centrifugation at 110,000 *g* for 15-16 h [6].

2. Lymphocytic cells

Separations of lytic particles containing Granzyme B and/or granulysin have generally been carried out in gradients containing 8-27% (w/v) iodixanol [7-9], centrifuged at 150,000 *g* for 5h. After homogenization, the post-nuclear supernatant from a lymphocytic cell suspension was centrifuged at 18,000 *g* for 20 min, to produce a light+heavy mitochondrial fraction, which was analyzed in the iodixanol gradient. In ref 8, which provides a detailed account of the methodology, the gradient was constructed from 8, 12, 16, 19, 22.5 and 27% (w/v) iodixanol and the sample was median loaded in the 19% (w/v) iodixanol layer. During the centrifugation the gradient will have become more or less linear. Granulysin was strongly enriched in a median and the densest band in the gradient. Median-loading can have a distinct advantage over top-loading: in the latter all the particles are sedimenting while in the former some will be moving up the gradient, others down, thus improving particle resolution. A slightly different continuous gradient of 6-30% (w/v) iodixanol at a lower *g*-force (100,000 *g* for 2h) was used by Kozlowski et al [10].

3. Neutrophils and HL60 cells (see ref 11)

HL60 cells were homogenized in a buffered HEPES-buffered 0.25 M sucrose medium containing 0.3 mM EDTA and mixed with an equal volume of iodixanol (1.12 g/ml density); layered between an equal volume of a

1.05 g/ml solution and 5 ml of the 1.12 g/ml solution. After centrifugation at 37,000 *g* for 35 min azurophilic granules (identified by syncollin) banded predominantly around the lower interface.

4. Neuromelanin granules (see ref 12)

Neuromelanin granules released from human brain synaptosomes were adjusted to 20% iodixanol and layered over a discontinuous 26%, 31%, 36% and 50% iodixanol gradient and centrifuged at 81,000 *g* for 3h, the granules banded on the 20-26% interface.

5. Parotid acinar cells (see ref 13)

Amylase-containing granules released from rat paraotid gland, homogenized in a routine isoosmotic Tris-buffered 0.25 M sucrose solution. The post-nuclear supernatant was layered on top of a continuous 10-30% (w/v) iodixanol gradient (solutions prepared by dilution of OptiPrep™ with homogenization medium) and centrifuged at 137,000 *g* for 2h: the granules banded around 20% (w/v) iodixanol.

6. Platelets

Platelets are most effectively homogenized by nitrogen cavitation [14] and the iodixanol gradients used for granule isolation, described in refs 14 and 15, are based on the use of this method . Homogenization of platelets is carried out in 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 0.5 mM EGTA, 25 mM Hepes-NaOH, pH 7.4. A 50% (w/v) iodixanol working solution is prepared from 5 vol. of OptiPrep ™ and 1 vol. of 145 mM NaCl, 30 mM KCl, 6 mM $MgSO_4$, 60 mM glucose, 3 mM EGTA, 25 mM Hepes-NaOH, pH 7.4. Further dilutions are made with the homogenization medium.

The cavitate is adjusted to 11% (w/v) iodixanol, transferred to a centrifuge tube for a swinging-bucket rotor and underlaid with a 30% (w/v) iodixanol solution. After centrifugation at 38,000 *g* for 3 h interfacial band is collected and dialyzed to remove the iodxanol.

A second gradient is prepared from 2 ml each of 1%, 15%, 20%, 25% and 30% (w/v) iodixanol; the dialyzed sample (approx 2.5 ml containing approx 2 mg protein/ml) layered on top and centrifuged at 38,000 *g* for 3 h. The rotor is allowed to decelerate from 2000 rpm without the brake (or a controlled deceleration program is used). It is not known if the band from the first gradient might be collected in the 30% iodixanol solution and used as the second gradient bottom layer. If the separation is based on density alone then this is a valid alternative; if on the other hand the separation is based on sedimentation rate, then such an approach is invalid. The α-granules band at the 25%/30% interface

7. References

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