Subcellular Fractionation: What You Need to Know (The rest is in books).

WHAT IS IT?

Functional studies of organelles and macromolecular complexes require detailed observation/manipulation
Impossible with whole cells.

Four great things about subfractionation:

1. Study single biological processes free from other interfering reactions in the cell w/o having to worry about keeping the cells alive-cell free system

2. Fractions are biologically active and can be stored

3. Minimal equipment needed and starting material is easily obtained

4. Protocols have already been worked out and are highly reproducible

Guiding questions for determining protocol:

1. **What's your goal?**
   a. **Do you need enzymatic activity?**
   b. **Are you looking at composition or morphology?**
   c. **Are you isolating a specific organellar protein?**

2. **What's your starting material? Tissue? Cultured cells, yeast, bacteria?**

*** Use the gentlest homogenization procedure for your cells to preserve function of organelles.**

What we've learned so far using this technique:

1. Mechanism of protein synthesis
2. DNA replication and transcription
3. RNA splicing
4. Muscle contraction
5. Microtubule assembly
6. Vesicular transport in the secretory pathway
7. Importance of mitachondria and chloroplasts in energy interconversions

Steps:

I. Homogenization
II. Differential centrifugation
III. Further separation and purification by density gradient centrifugation
IV. Collection of fractions
V. Analysis of fractions

Equipment:

Low speed ultracentrifuge w/ rotors
High speed ultracentrifuge w/ rotors
Spectrophotometer (measuring protein concentration)
Method to evaluate fractions

Rotors:

Fixed angle or swinging bucket

Homogenization

1. Osmotic shock-make the cells swell/explode in hypo-osmotic buffer (cultured cells)

2. Sonication- break open cells with sound waves

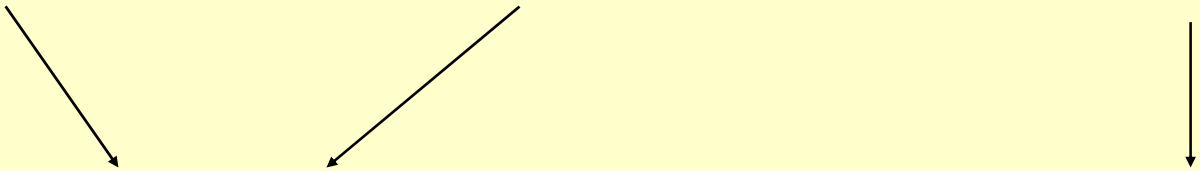
3. Mechanical exploding/shearing/grinding- Nitrogen cavitation devices, blenders, Dounce homogenizers, Potter-Elvehjem teflon homogenizers (whole tissue)

- Buffers are usually isotonic, contain some sucrose, protease inhibitors, and are pH 7.4.
- *Everything done on ice and at 4°C!*

Protocols: Maniatis, Spector Goldman and Leinwand, Amersham Pharmacia

Centrifugation rotors

vertical **fixed angle** **swinging bucket**



Orientation of solution in tube horizontal to vertical during acceleration…good for density gradients b/c equilibrium achieved quickly with the short path

Orientation of the solution in tube vertical to horizontal during acceleration…fractions concentrate to outer edges of tube walls

Carried out at 4°C, transfer of material is with wetted glass pipettes or wetted syringes

Centrifugations in Subcellular Fractionation:

Differential centrifugation- separates particles on the basis of size and yields crude fractions
-usually first step in fractionation

Density gradient (isopycnic) centrifugation- separates particles on the basis of density and size, and yields purer organellar fractions

Particle behavior in a centrifugal force

Particles in suspension can be separated by either **sedimentation velocity**, (differential centrif.) or by **sedimentation equilibrium** (isopycnic or density centrif.). Their separation depends on:

Sedimentation Velocity

Sedimentation Coefficient (S for Svedberg)

Diffusion Coefficient

What is important is that S can be measured and will give an important clue as to the physical structure and size of the particle.

The sedimentation coefficient is given by the formula:

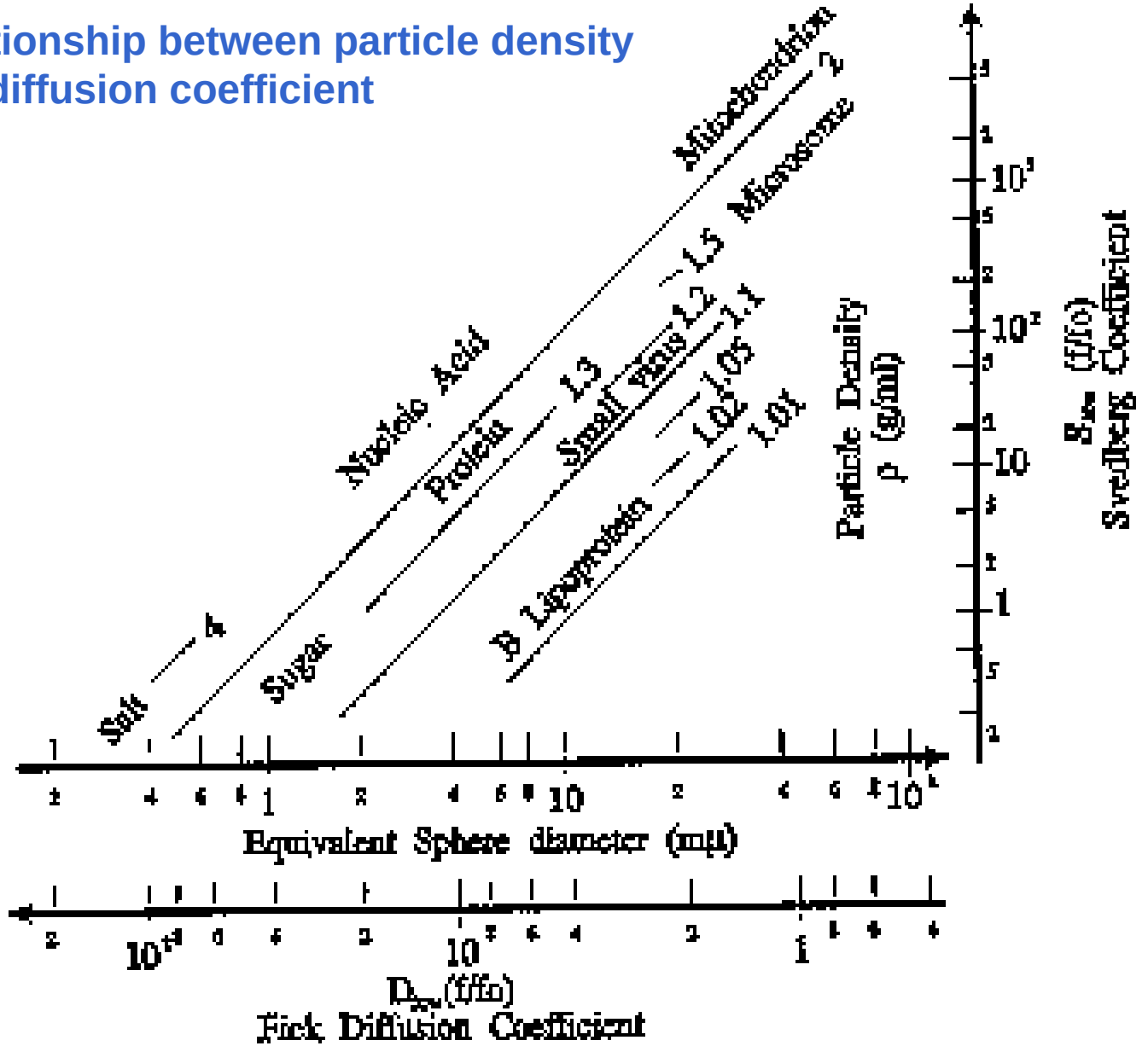
$S = 1/ \omega^2 r \times dr/dt$

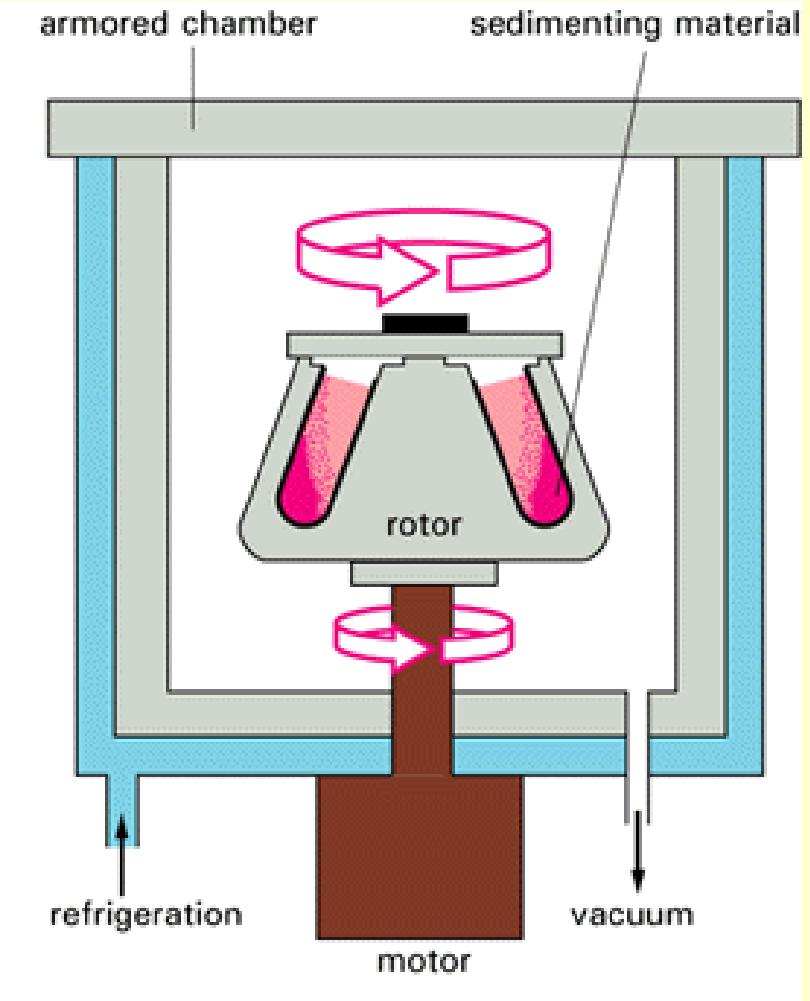
ω= angular velocity of the rotor in radians/sec calculated as 0.10472 x RPM

r = the distance between the particle and the center of rotation (mm)

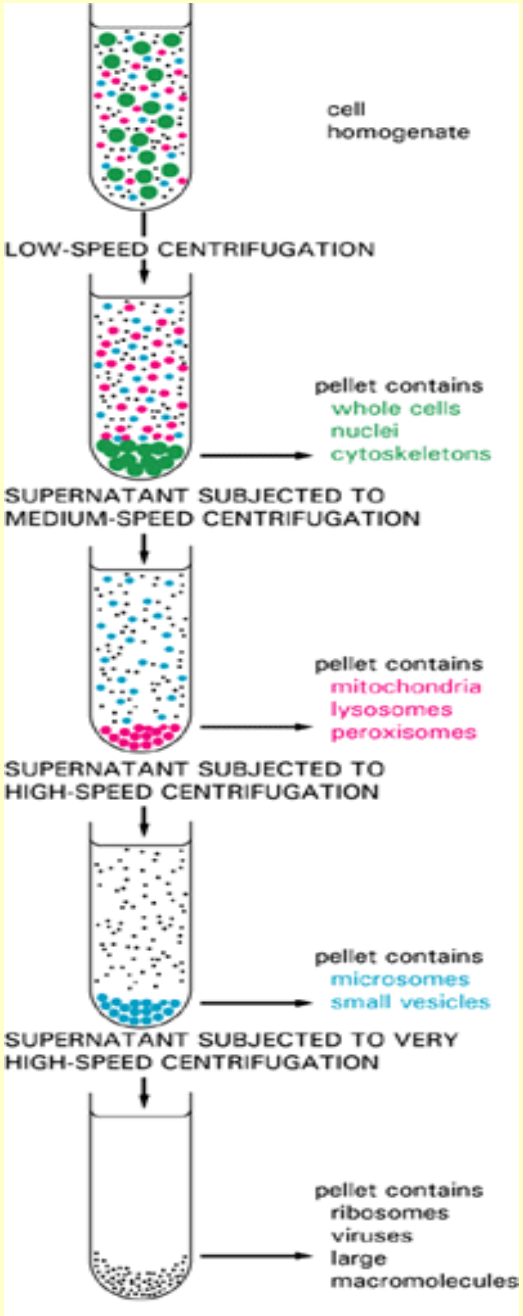
dr/dt = the rate of movement of the particle (cm/sec)

Relationship between particle density and diffusion coefficient





The preparative ultracentrifuge. Sample is contained in tubes that are inserted into a ring of cylindrical holes in a metal *rotor*. Rapid rotation of the rotor generates enormous centrifugal forces, which cause particles in the sample to sediment. The vacuum reduces friction, preventing heating of the rotor and allowing the refrigeration system to maintain the sample at 4°C.



Differential Centrifugation

Repeated centrifugation at progressively higher speeds will fractionate homogenates of cells into their components. Values for the various centrifugation steps referred to in the figure are:

low speed: 1,000 times gravity for 10 minutes

medium speed: 20,000 times gravity for 20 minutes

high speed: 80,000 times gravity for 1 hour

very high speed: 150,000 times gravity for 3 hours

Differential centrifugation crude pellets from rat liver

Pellet	RCF x time	Content
P1	1,000g x 10m	nuclei, heavy mitochondria, PM sheets
P2	3,000g x 10m	heavy mitochondria, PM fragments
P3	6,000g x 10m	mitochondria, lysosomes, peroxisomes, intact Golgi
P4	10,000g x 10m	mitochondria, lysosomes, peroxisomes, Golgi membranes
P5	20,000g x 10m	lysosomes, peroxisomes, Golgi, large and dense vesicles (rER)
P6	100,000g x 10m	all ER vesicles, PM, Golgi, endosomes

Size and Sed. Properties for Differential Centrifugation

TABLE 34.2
Size and sedimentation properties of subcellular components

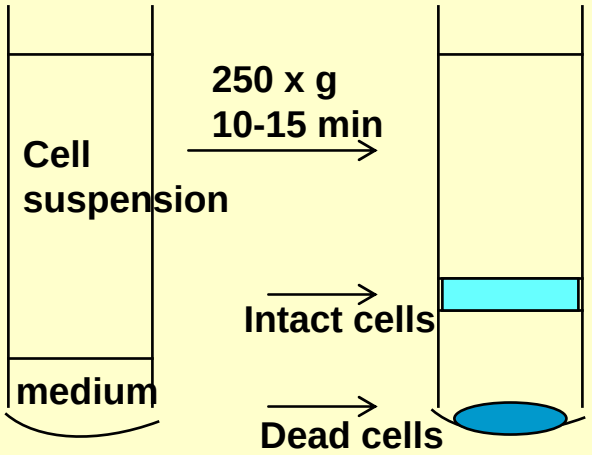
Subcellular component	Size (μm)	RCF (g_{av})[a]	Time (min)
Nucleus	4–12	500–1,000	5–10
Nuclear membrane[b]		2,000 (30,000)	30 (5)
Mitochondria	0.4–2.5	1,000–10,000	10–15
Lysosomes	0.4–0.8	6,000–15,000	10–20
Peroxisomes	0.4–0.8	6,000–15,000	10–20
Rough ER vesicle[c]	0.05–0.35	30,000–100,000	30–60
Smooth ER vesicle[c]	0.05–0.3	50,000–100,000	30–60
Plasma membrane			
sheet	3–20	1,000–3,000	10–15
vesicles	0.05–2.0	50,000–100,000	30–60
Endosome	0.05–0.4	50,000–100,000	30–60
Golgi (intact)	1.0–2.0	10,000–20,000	20–30
Golgi (vesicle)	0.05–0.5	50,000–100,000	20–40
Sarcoplasmic reticulum	0.1–1.0	10,000–35,000	20
Chloroplasts	2–5	1,000–2,000	10
Plant mitochondria	1–3	5,000–20,000	15

(Reprinted, with permission, from Dealtry and Rickwood 1992.)
[a]RCF = relative centrifugal force.
[b]Nuclear membrane preparations are intact envelopes (ghosts), whose sedimentation rate depends on the mode of preparation.
[c]ER = endoplasmic reticulum.

Taken from *Cells, A Laboratory Manual.* Spector, Goldman, & Leinwand

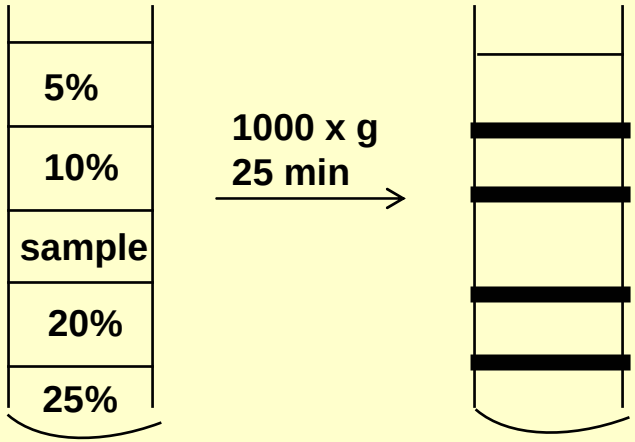
Separation of cells before subfractionation

A.



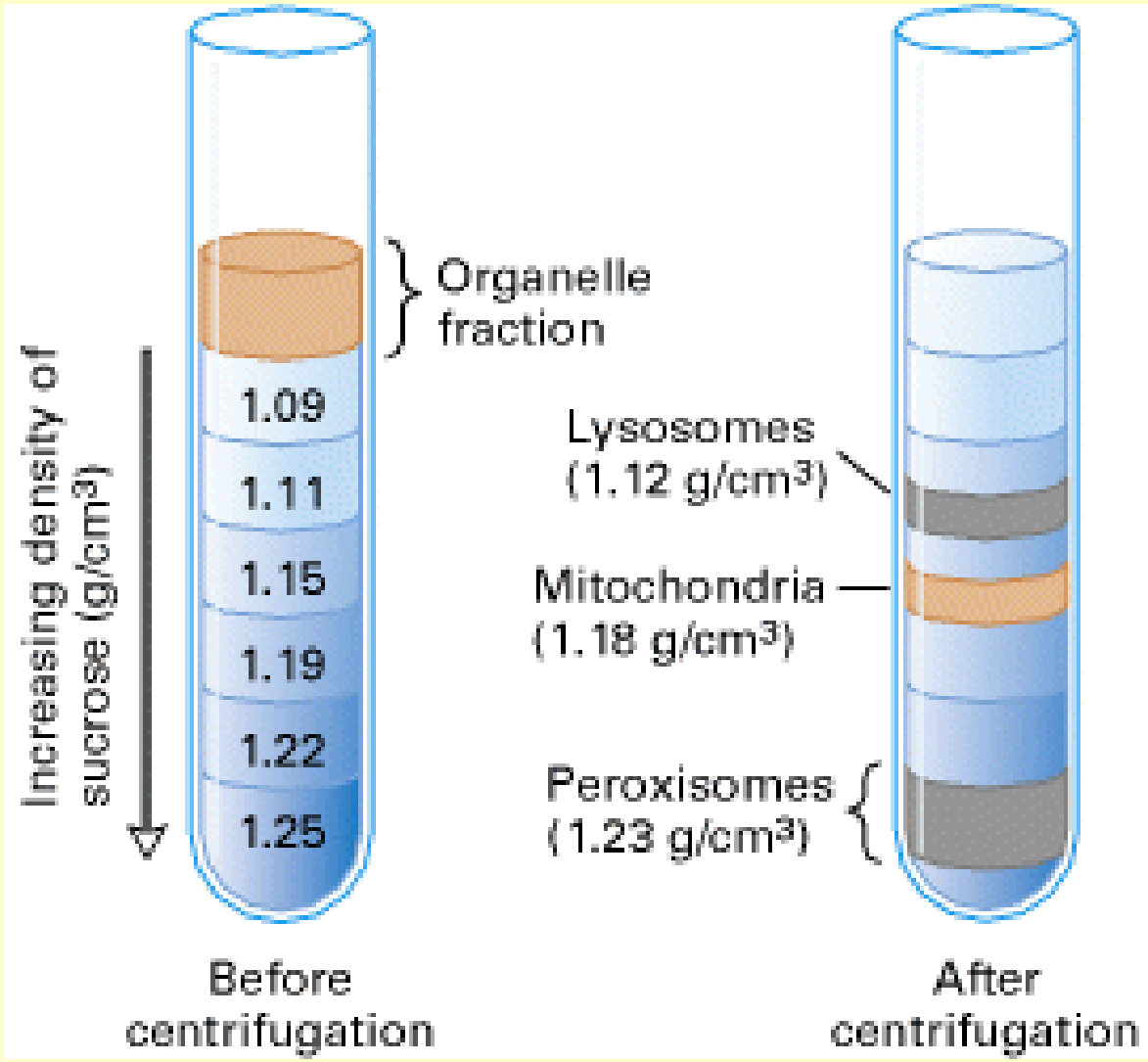
Separation of intact and damaged cells.

B.



Separation of different kinds of cells (cells migrate to gradient interfaces).

Density Gradient Centrifugation (sep. based on density)



The type of medium for density centrifugation is dictated by the goal of the fractionation.

Media

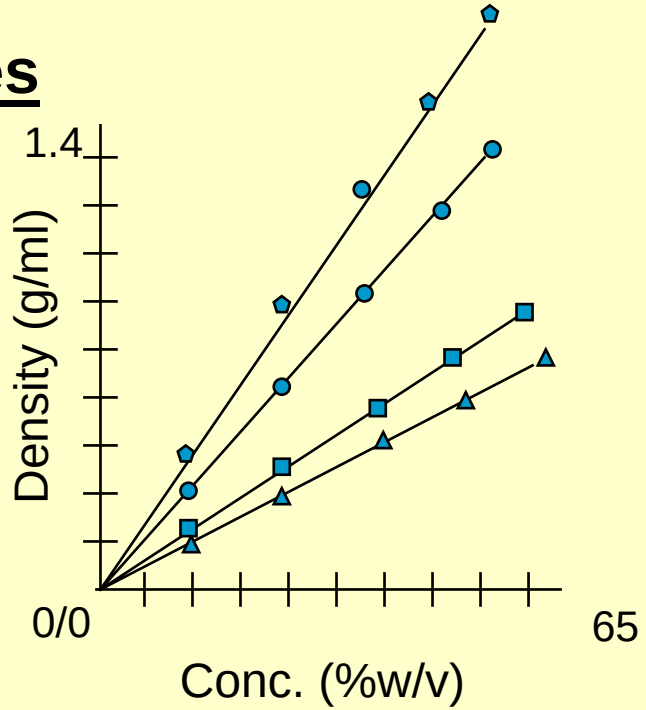
Sucrose cheap, soluble, broad range of densities can be prepared that are required for separating most organelles
BUT: very viscous and hyperosmotic at high conc.

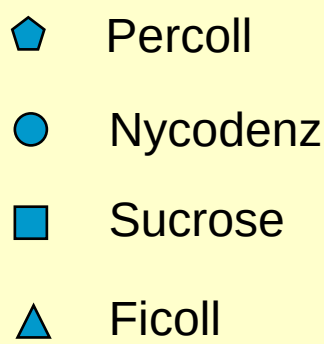
Nycodenz non-ionic, iodinated derivative of tri-iodobenzoic acid, provides lower osmolalities for a broad range of densities, soluble in most aq. media, stable over broad pH range
BUT: Expensive

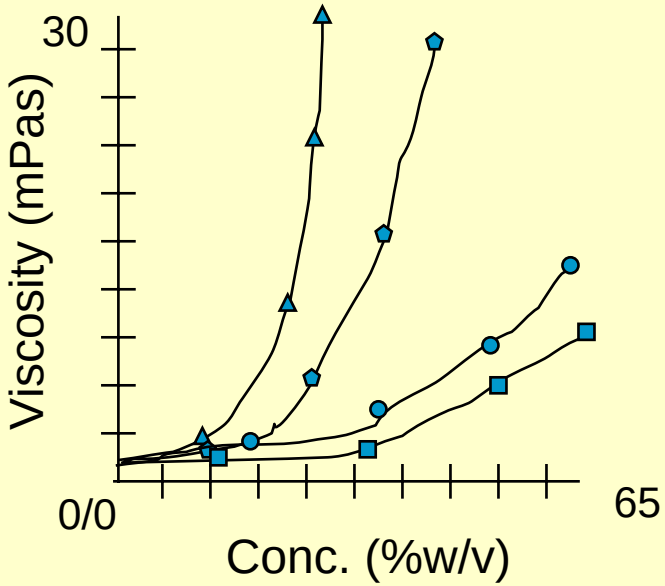
Percoll coated colloidal silica, no osmotic effect, simple to use, mix sample with Percoll buffer and it will form own gradient during centrifugation in a fixed angle rotor
BUT: Percoll particles sediment rapidly, so it's no good at higher speeds

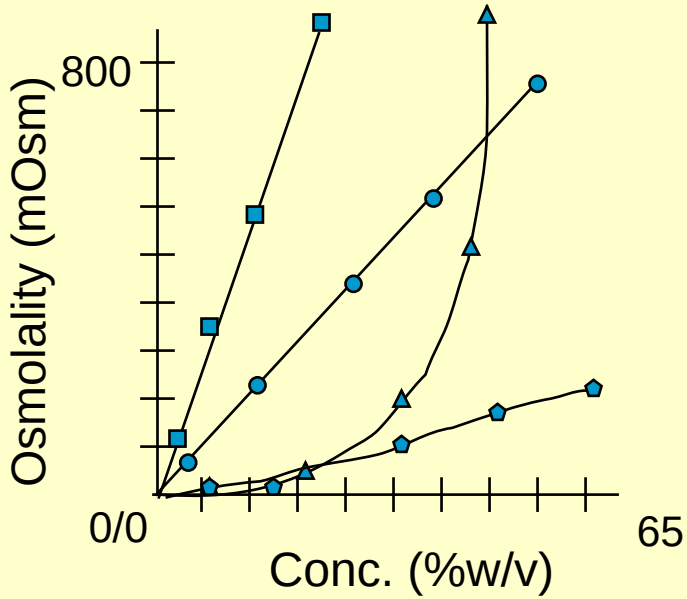
Ficoll 400 polymer of sucrose and epichlorohydrin, low osmol. at low conc., but osmolality increases dramatically above concentrations of 30%
BUT: very viscous at concentrations above 10%

Media properties









Physical Characteristics of Different Media

Medium	Concentration (%w/v)	Density (g/ml)	Viscosity (cP)	Osmolality (mOs/kg H_2O)
Sucrose	20	1.06	30	700
Metrizamide™	30	1.16	2	260
Ficoll™	30	1.10	49	130
Percoll	26	1.13	10	10

Non-ionic, stable, inert

→ Nycodenz/Accudenz and Iodixanol

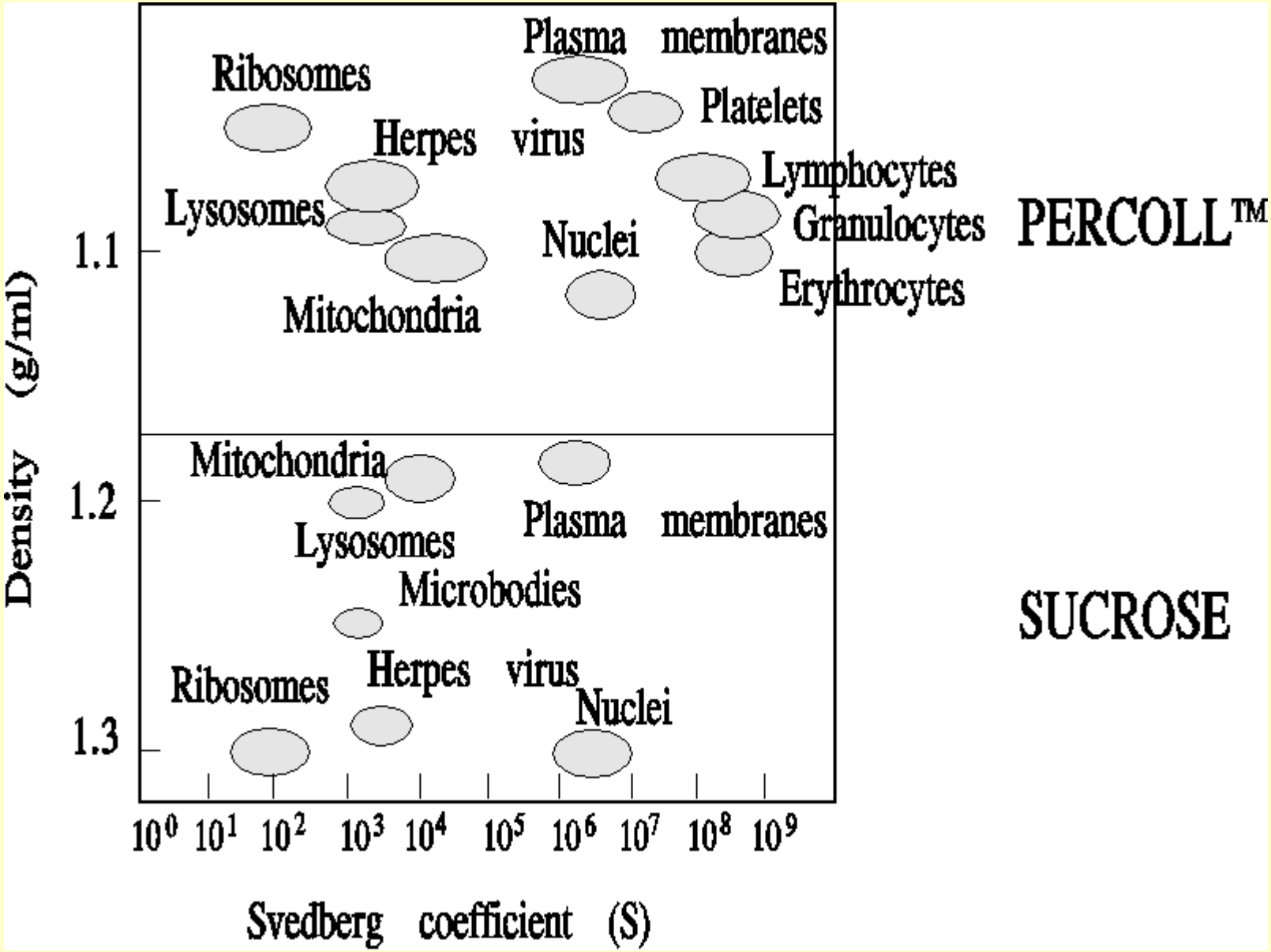
Densities of organelles

TABLE 34.3
Density of subcellular components in sucrose and low osmolarity media

Subcellular component	Density ($g\ cm^{-3}$)	
	sucrose	low osmolarity medium[a]
Nucleus	>1.30	1.21–1.24
Nuclear membrane	1.18–1.22	n.a.
Mitochondria	1.17–1.21	1.15–1.20
Mitoplasts	1.14	n.a.
Lysosomes	1.19–1.21	1.10–1.15
Peroxisomes	1.18–1.23	1.19–1.22
Rough ER	1.18–1.26	n.a.
Smooth ER	1.06–1.15	1.03–1.07
Plasma membrane		
sheets	1.14–1.19	1.12–1.15
vesicles	1.07–1.16	1.05–1.12
Endosomes	1.06–1.16	1.05–1.10
Golgi (intact)	1.05–1.12	1.03–1.08
Golgi (vesicle)	1.05–1.12	1.03–1.08
Sarcoplasmic reticulum	1.04–1.08	n.a.
Chloroplasts	1.18–1.20	1.10–1.13
Plant mitochondria	1.16–1.19	n.a.

n.a. = not available. (Reprinted, with permission, from Dealtry and Rickwood 1992.)
[a]These figures are densities in Nycodenz™, metrizamide, or Percoll™.

Taken from *Cells, A Laboratory Manual.* Spector, Goldman, & Leinwand


Plasma membranes
Ribosomes
Platelets
Herpes virus
Lymphocytes
Lysosomes
Granulocytes
PERCOLL™
Nuclei
1.1
Erythrocytes
Mitochondria
Density (g/ml)
Mitochondria
1.2
Plasma membranes
Lysosomes
Microbodies
SUCROSE
Ribosomes
Herpes virus
Nuclei
1.3
10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9
Svedberg coefficient (S)

Types of gradient media for different isolations

TABLE 34.4
Recommended gradient and centrifugation conditions for purification of subcellular components

Component[a]	Pellet[a]	RCF (g_{av}) x time	Gradient[a]	Contaminants[a]	References
NUC	P1	100,000*g* x 60 min	60% sucrose barrier	none	Blobel and Potter 1966; Comerford et al.
	P1/HOM	15,000*g* x 60 min	20–50% dc Nycodenz™	none	1985; Graham et al. 1990
PMS	P1	160,000*g* x 3 hr	37%/60% sucrose	MIT	Graham 1984
MIT	P2	3,000*g* x 10 min	none	NUC	
	P2+P3+P4	50,000*g* x 2 hr	20–40% dc Nycodenz™	PER	Graham et al. 1990
	HOM	37,000*g* x 30 sec	20–52% dc Percoll™	PER	Graham 1984
LYS	P4+P5	50,000*g* x 2 hr	10–50% dc Nycodenz™	PM	Graham et al. 1990
	P4+P5	37,000*g* x 30 min	15–85% c Percoll™	MIT	Graham 1984
PER	P4+P5	95,000*g* x 2 hr	34–47% dc Nycodenz™	MIT	Wattiaux and Wattiaux-De Coninck 1983
GOLGI	P4+P5	160,000*g* x 1 hr	10–44% dc sucrose	PMV/SER	Graham 1984
	P4+P5	50,000*g* x 2 hr	10–50% dc Nycodenz™	LYS	Graham et al. 1990
SER/RER	P6	150,000*g* x 1 hr	20%/45% sucrose with 15 mM CsCl	PMV/END	Bergstrand and Dallner 1986
PMV[b]	P4+P5/P6	100,000*g* x 4 hr	10–30% c metrizamide	SER/END	Graham and Winterbourne 1988
END[c]	HOM/P6	85,000*g* x 45 min	5–35% c Nycodenz™	SER/PMV	Kindberg et al. 1984
SARCRT	P2	120,000*g* x 4 hr	20–38% dc sucrose	MIT	Meissner 1984
NUCMB[a]	P1	100,000*g* x 1 hr	10–55% dc sucrose	none	Kay et al. 1972; Graham et al. 1990
OUTER MITMB[d]	P2	115,000*g* x 1 hr	23–43% dc sucrose	INNER MITMB	Coty et al. 1979

(Reprinted, with permission, from Dealtry and Rickwood 1992.)
[a]Abbreviations: (NUC)Nuclei; (PMS) plasma membrane sheets; (MIT) Mitochondria; (LYS) lysosomes; (PER) peroxisomes; (PMV) Plasma membrane vesicles; (SER) smooth endoplasmic reticulum; (RER) rough endoplasmic reticulum; (END) endosomes; (SARCRT) sarcoplasmic reticulum; (NUCMB) nuclear membrane; (OUTER MITMB) outer mitochondrial membrane; (INNER MITMB) inner mitochondrial membrane; (HOM) homogenate; (dc) discontinuous; (c) continuous.
[b]Method can be used for the fractionation of secretory vesicles.
[c]Method can be used for the fractionation of any vesicle on the basis of size.
[d]Pellets require pretreatment before application to the gradient (see Coty et al. 1972; Kay et al. 1972; Graham et al. 1990).

Taken from *Cells, A Laboratory Manual.* Spector, Goldman, & Leinwand

Making gradients by hand:

1. **Underlay each layer**

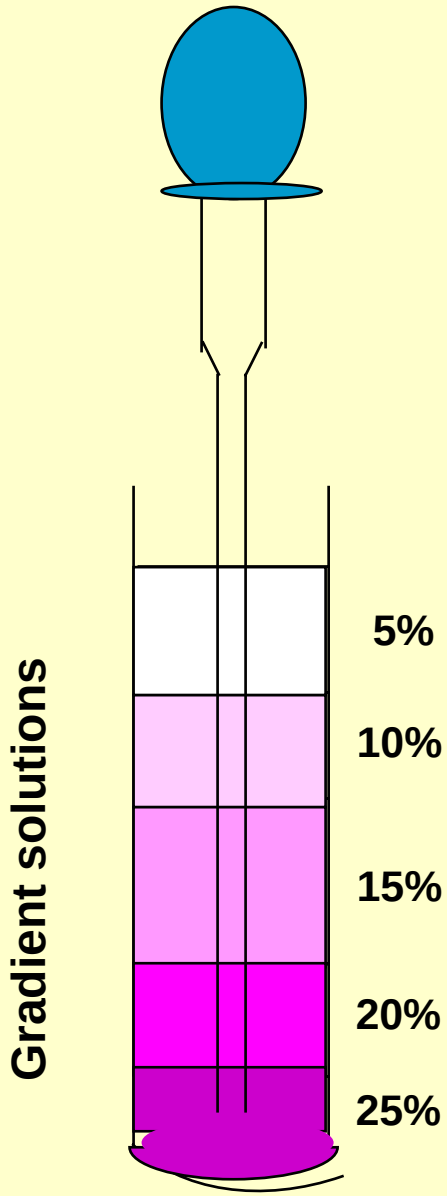
2. **Fraction/sample generally should be loaded at bottom of centrifuge tube**

3. **Make gradients isoosmotic!**

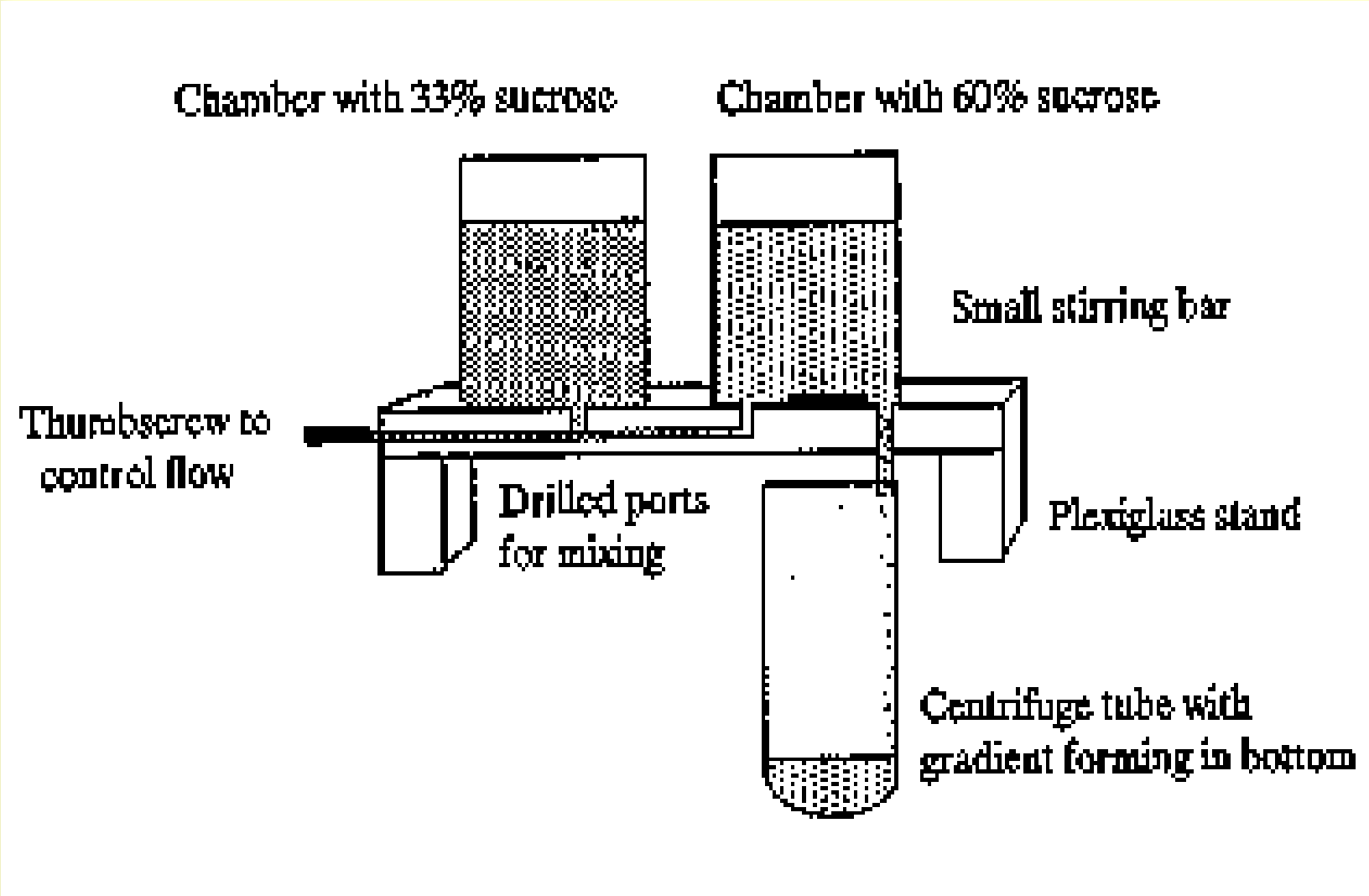
Pre-formed v. self-forming

Swinging bucket
Fast sedimenting

Fixed angle
Slow sedimenting



Device for Creating Gradients



Collecting Fractions- keeping samples pure and intact

1. By hand: puncture sidewall of centrifuge tube with needle and withdraw fractions through syringe

2. Machine: gradient uploader; introduces very dense, non-miscible medium into bottom of tube, pushes fractions up to be collected from top

3. If no pellet, can collect fractions through hole in bottom of tube

****** USE METHOD WHICH CAUSES LEAST DISTURBANCE TO GRADIENT AND SAMPLES!***

Analysis of fractions- need to identify and quantify the purified fractions, so that they can be used successfully in downstream applications

Methods:

1. Light or electron microscopy

2. Biochemical-determine presence of marker enzymes

3. Assay for a protein marker with an antibody (western)

4. Determine the protein concentration by using a spectrophotometer, e.g. Bradford assay

5. Determine specific activity (the ratio of activity of the enzyme of interest to the protein concentration

Enzyme assays to identify organelles in fractions

Organelle	Hallmark enzyme
1. ER	NADPH-cytochrome c reductase
2. Golgi	Galactosyl transferase
3. Lysosomes	β-Galactosidase
4. Mitochondria	Succinate dehydrogenase
5. Peroxisomes	Catalase
6. Plasma membrane	5’ Nucleotidase

See table 34.5 in handout

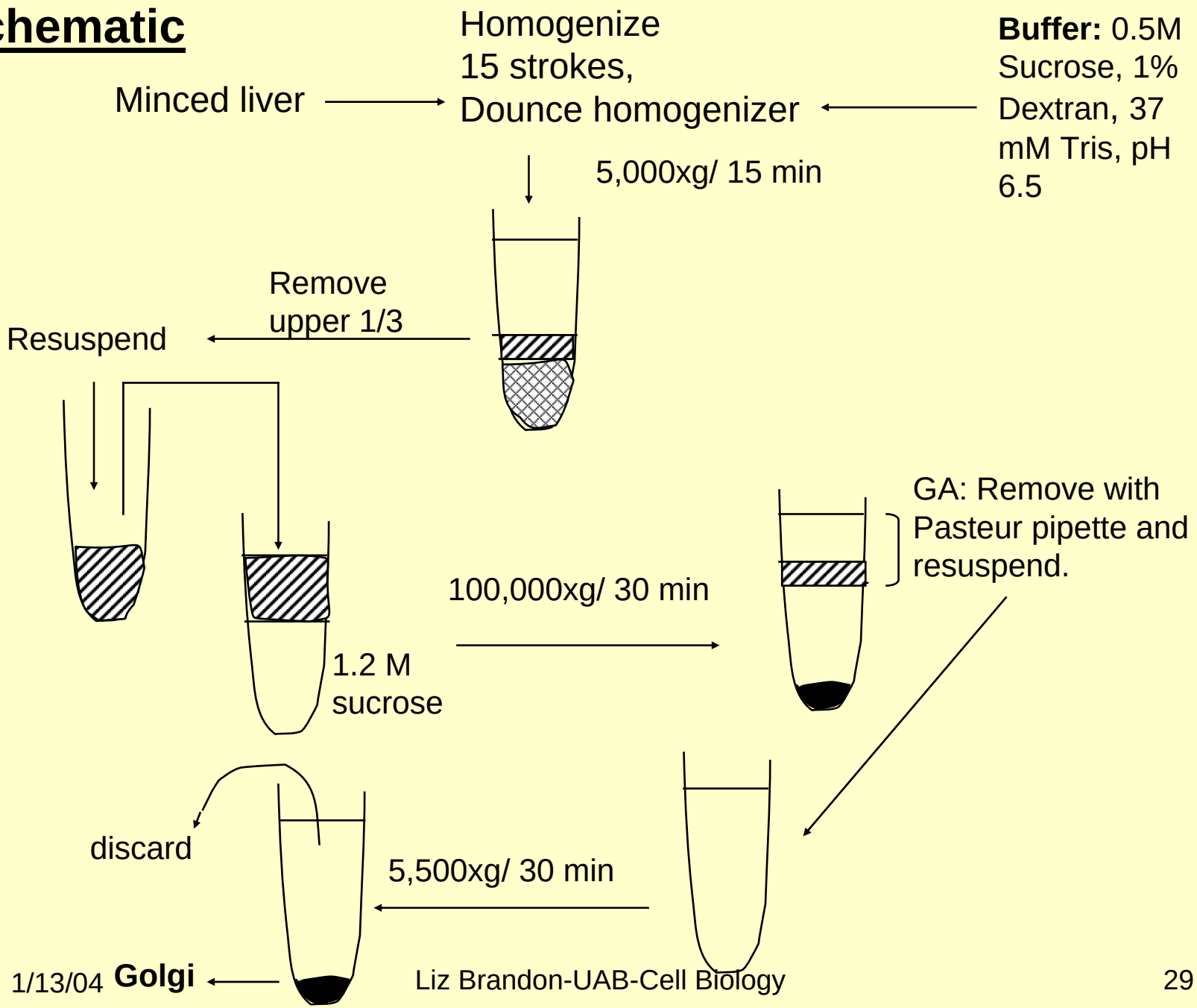
Subfractionation of rat liver, Sztul lab

Goal: isolation of Golgi

Media: Sucrose, won't affect proteins, cheap and readily available

Analysis: will identify fractions by Western for marker proteins

Schematic


Minced liver
Homogenize
15 strokes,
Dounce homogenizer
Buffer: 0.5M Sucrose, 1% Dextran, 37 mM Tris, pH 6.5
5,000xg/ 15 min
Remove upper 1/3
Resuspend
1.2 M sucrose
100,000xg/ 30 min
GA: Remove with Pasteur pipette and resuspend.
discard
5,500xg/ 30 min
Golgi

Plasma membrane

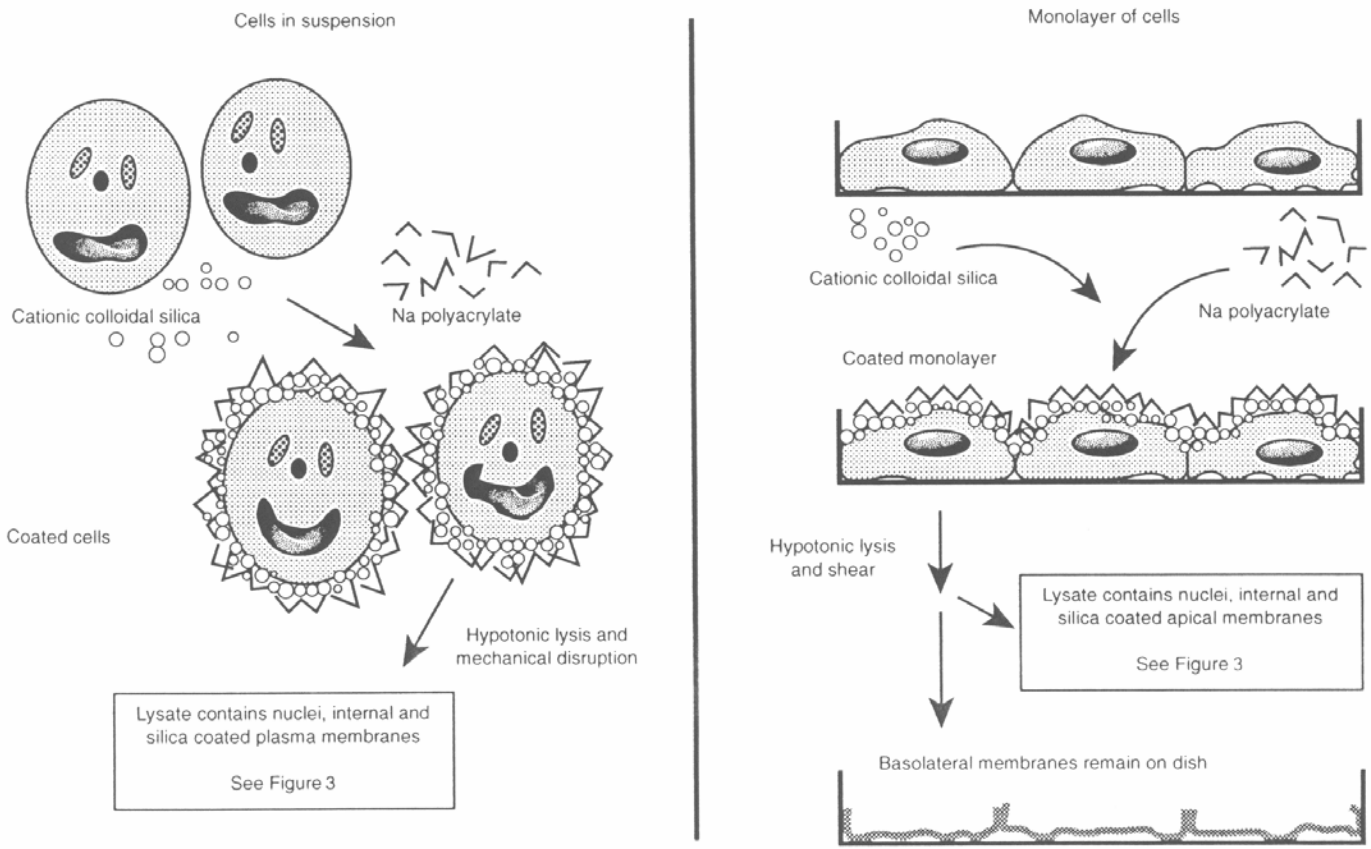


FIGURE 35.2
Silica coating and polyacrylate overcoating of cell surfaces when cells are in suspension (*left*) or are growing as monolayers in culture dishes (*right*).

35.3

Taken from *Cells, A Laboratory Manual.* Spector, Goldman, & Leinwand

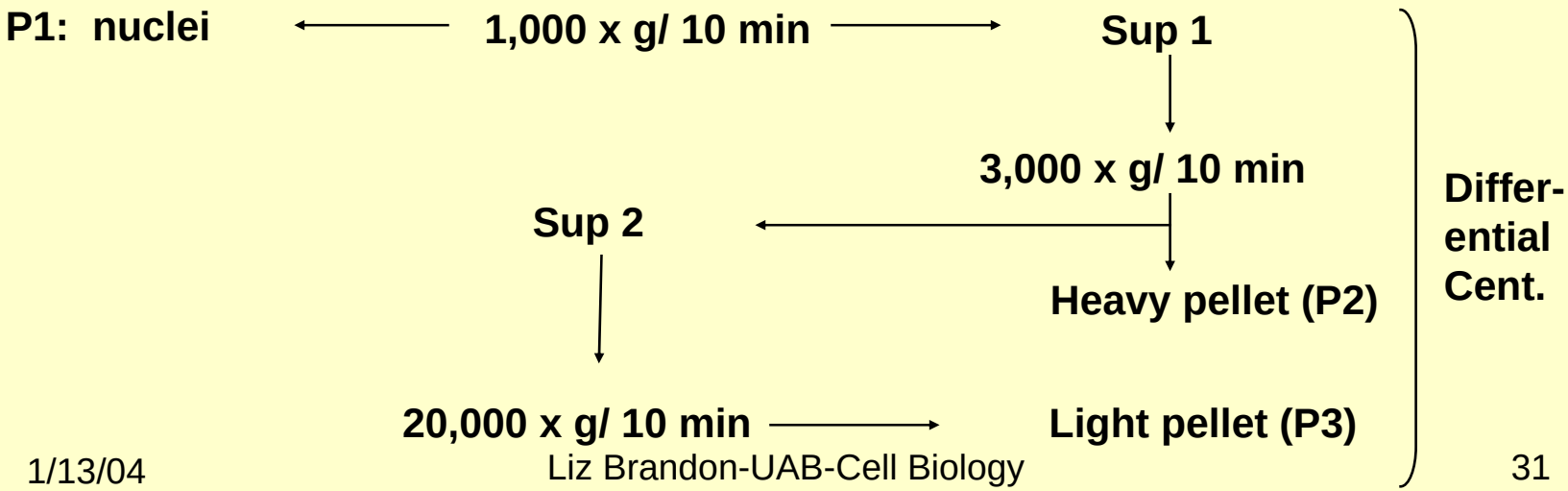
A Basic Procedure with Nycodenz:

1. **Sacrifice rat by cervical dislocation**
2. **Excise liver and wash several times with ice-cold sucrose buffer**
3. **Mince with scalpel, 10-12 strokes with Dounce homogenizer**
4. **Divide into one small (nuclei) and one larger aliquot (organelles)**
5. **Filter nuclear aliquot through nylon filter to get rid of particulate matter**
6. **Nuclear**

(buffer: 8% sucrose, 25 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-base, pH 7.8)

Other organelles

(buffer: 8% sucrose, 1 mM EDTA, 20 mM Tris-base, pH 7.8)



7. **Resuspend pellets in buffer ⟶ on ice.**

Separating ER/peroxisomes/lysosomes

1. **Make 10 mL of 10-30% Nycodenz gradient (discontinuous)**
2. **Bring P3 (light mitochondrial fraction) to 35% Nycodenz (5-6 mL)**
3. **Underlay each gradient layer w/ 1-2 mL of sample**
4. **Centrifuge at 50,000 x g/ 1.5 hr/ 4°C/ swinging bucket rotor**
5. **Collect fractions in 0.5 mL aliquots**
6. **Analyze by Western blot**

Re-cap of Subcellular Fractionation

- **What technique is used for**
- **How to pick the best protocol (guiding questions)**
- **Types of density gradient media**
- **Basic procedure (H, D, D, C, A) Hot Damn Day, Cold Amstel**