Chapter 5

Hydrodynamic Analysis of Human Kinetochore Complexes During Mitosis

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Abstract

Hydrodynamic analysis is a powerful tool to dissect the molecular architecture of macromolecular protein assemblies. These techniques have been successfully used in yeast systems but are also well suited to the analysis of protein complexes from human cells. Furthermore, the combination of hydrodynamic analysis with siRNA mediated protein depletion provides an excellent system to probe the composition of protein complexes isolated from human cells. In this chapter we describe the use of these approaches in the analysis of macromolecular protein complexes during mitosis in human cells, using the kinetochore as an example.

Key words: Size-exclusion chromatography, sedimentation equilibrium, glycerol gradient, Svedberg coefficient, Stokes radius, kinetochore, mitosis.

1. Introduction

The human kinetochore is a large proteinaceous structure ~80–100 nm thick and 0.5–1.0 μm in diameter that assembles on centromeric DNA, biorients paired sister chromatids on spindle microtubules, and controls anaphase onset via the spindle checkpoint *(1)*. This complexity of function is reflected in the extraordinary structural complexity of the human kinetochore; to date, affinity purification and sequence search-based approaches have identified more than 80 kinetochore proteins. The combination of affinity purification and mass spectroscopy is an excellent approach to identify novel factors, and can reveal protein interaction networks. Such networks, however, do not distinguish

Andrew D. McAinsh (ed.), *Mitosis: Methods and Protocols*, *vol. 545*

DOI 10.1007/978-1-60327-993-2_5, Springerprotocols.com

clearly between proteins that are present in stable complexes and those that interact in a more transient fashion. Biochemical analysis using size-exclusion chromatography and glycerol gradients has proved a powerful approach to discriminate between these possibilities *(2)*.

Hydrodynamic analysis relies on the combination of two classical techniques: Sedimentation equilibrium analysis (specifically, glycerol gradient ultracentrifugation) and size-exclusion chromatography. Both these methods separate proteins or complexes based on their molecular weight and shape. Nonglobular proteins or complexes behave differently to the globular standards used to calibrate both sedimentation equilibrium analysis and size-exclusion chromatography systems, giving rise to inaccurate estimations of native molecular weight if only one technique is employed. Using information from both techniques not only allows a more accurate measurement of the molecular weight of a protein or complex, but also provides information about the degree to which a complex is distorted from a globular state. These techniques are equally effective in the analysis of either native complexes from cell extracts or recombinant purified proteins. Although the methodology to analyse yeast extracts has been described *(3)*, here we provide detailed step-by-step instructions to allow any scientific researcher to perform the experiments and calculations necessary to probe the organisation of proteins and multiprotein assemblies in human mitotic cell extracts. Moreover, we show how combining this hydrodynamic analysis with siRNA-mediated protein depletion can be a powerful approach to probe the subunit composition of multiprotein complexes.

2. Materials

All chemicals are from Sigma-Aldrich, unless otherwise stated.

2.1. Cell Extract Preparation

1. H100 buffer (*see* **Note 1**; 50 mM HEPES pH 7.9, 1 mM EDTA, 100 mM KCl (*see* **Note 2**), 10% glycerol, 1 mM $MgCl_2$, 50 mM B-glycerolphosphate, 10 mM NaF, 0.25 mM $NaOVO_3$, 5 nM okadaic acid, 5 nM Calyculin A). Filter-sterilise and freeze in aliquots. On day of use add protease inhibitors (Complete (Roche)) and phosphatase inhibitors (Phophatase inhibitor cocktails A and B,(Sigma)).
2. Phosphate buffered saline (PBS).
3. Nocodazole (final concentration of 100 ng/ml; *see* **Note 3**).
4. Double distilled H_2O (ddH_2O).
5. 50 ml Falcon tubes.
6. Liquid nitrogen.

7. Porcelain mortar and pestle (Coors; Sigma).
8. High-speed ultracentrifuge, for example, Sorvall Ultra Pro 80.
9. Bradford Assay reagents or spectrophotometer system, for example, Nanodrop (Thermo Scientific).

2.2. Sedimentation Equilibrium Analysis; Glycerol Gradients

1. 2 × K150 buffer (100 mM HEPES pH 7.9, 2 mM EGTA pH 8, 2 mM $MgCl_2$, 600 mM KCl)
2. K150 + 5 % glycerol (5% (v/v) glycerol, 50% 2 × K150 buffer, 45% (v/v) ddH_2O)
3. K150 + 40 % glycerol (40% (v/v) glycerol, 50% 2 × K150 buffer, 10% (v/v) ddH_2O)
4. 13 × 51 mm polyallomer tubes (Beckman)
5. Optional: Gradient Master (Biocomp)

2.3. TCA Precipitation

1. Trichloroacetic acid (TCA)
2. Acetone
3. SDS-loading buffer (150 mM Tris.HCl pH 6.8, 1.2 % (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) Beta-mercaptoethanol, 1.8 μg/ml Bromophenol blue)

2.4. Size-Exclusion Chromatography

1. K150 buffer (50 mM HEPES pH 7.9, 1 mM EGTA pH 8, 1 mM $MgCl_2$, 150 mM KCl; *see* **Note 4**).
2. Sephacryl size-exclusion chromatography column such as Pharmacia S-500 HR (Amersham).
3. High-pressure pump chromatography system such as Akta Purifier (Pharmacia).
4. Molecular weight globular protein standards (Amersham).

2.5. siRNA-Mediated Protein Depletion

1. Oligofectamine (Invitrogen).
2. siRNA oligos (Invitrogen/Qiagen).
3. Optimem (*see* **Note 5**; Invitrogen).
4. MEM media + 10 % FBS + 1% Penicillin/Streptomycin (all Invitrogen).
5. DMEM media + 10 % FBS + 1% Penicillin/Streptomycin (all Invitrogen).
6. 0.5 % Trypsin-EDTA (Invitrogen).

3. Methods

3.1. Making Mitotic Human Cell Extracts

In order to investigate the protein complexes involved in kinetochore function during mitosis we prepare our cell extract from human mitotic cells (*see* **Note 6**). Cells treated with the microtubule depolymerising drug, nocodazole, arrest in prometaphase due to lack of proper kinetochore–microtubule interactions

leading to the sustained activation of the spindle checkpoint. Because mammalian cells 'round up' during mitosis, they are less strongly attached to the base of the dish and can be shaken loose. This allows us to further enrich for mitotic cells which can then be broken open to release the soluble protein contents. The main methods used to make concentrated soluble protein extracts from mammalian cells include freeze thawing, dounce homogenising, and liquid nitrogen grinding. We use the latter as it is a robust technique which produces protein extract of high concentration, typically around 30 mg/ml.

1. Grow cells in 15 cm dishes to 70–80% confluency (*see* **Note 7**).
2. Aspirate medium from cells and replace with medium containing nocodazole to a final concentration of 100 ng/ml (*see* **Note 3**).
3. 14–16 h later check the mitotic index using phase contrast microscopy to count the percentage of mitotic (rounded up) cells. There should be about 60–70% mitotic cells (*see* **Note 3**).
4. Perform mitotic shake-off: use a tip box to firmly tap all around the side of the dish. After 10–20 taps check that most mitotic cells have been detached using phase contrast microscopy. Repeat shake-off if necessary.
5. Using a 25 ml pipette take up the media, wash once over the dish, take up again, and collect in 50 ml Falcon tubes.
6. Spin cells down at 130 g for 5 min at 4°C, wash once with 50 ml PBS, spin cells down again, and remove medium.
7. Weigh the wet cell pellet (keep on ice) and resuspend in 1.5 volumes H100 buffer (+ protease inhibitor cocktail and phosphatase inhibitor cocktails added on day of use; *see* **Note 8**).

   Warning: The next steps are hazardous and should be performed in accordance with the local safety regulations (i.e., safety glasses and cold-proof gloves).

8. Chill mortar and pestle with three rounds of liquid nitrogen boiling. Freeze the mortar to the bench by adding some water around the base.
9. Drip cell suspension into liquid nitrogen-filled mortar and break pellets into a powder with pestle.
10. Grind pellets in a circular motion as the liquid nitrogen boils away, and really grind the powder once the liquid nitrogen has completely gone. Take care to scrape powder off the side of the wall with a precooled metal spatula.
11. Repeat liquid nitrogen boiling/grinding once for a total two rounds (*see* **Note 9**).
12. After last round of grinding, scoop powder out of the mortar with a precooled spatula and transfer to cooled 1.5 ml Eppendorf tubes.

13. Spin at 15.7 g in a bench-top centrifuge for 30 min at 4°C.
14. Remove supernatant, place in appropriate cooled tube (e.g., Beckman 1.5 ml tube for TLA 45 rotor) for the high-speed spin and add KCl to a final concentration of 150 mM (*see* **Note 10**), and NP40 to 0.05%, and spin at 125,000 g for 30 min at 4°C.
15. Remove supernatant = final extract. Determine the protein concentration using either Bradford assay or spectrophotometry then aliquot and freeze at –80°C. A typical yield should be in the range of 20–30 mg/ml protein.

3.2. Sedimentation Equilibrium Analysis; Glycerol Gradients

Sedimentation equilibrium analysis involves the centrifugal force-driven movement of protein complexes through a glycerol or sucrose gradient. Protein complexes migrate through the gradient at a rate dependent on two parameters: the molecular weight of the complex and the elongation of the complex. Elongated complexes do not move through the gradient as quickly as a globular counterpart of the same molecular weight and so will behave as a globular complex of smaller molecular weight. Glycerol gradients are a simple technique for estimating the sedimentation coefficient (Svedberg unit or S value) of proteins or protein complexes. The sample is centrifuged through a gradient of glycerol, which provides a near-constant rate of movement through the gradient. As the proteins move farther away from the centre of the rotor they experience increased centrifugal force, which is counteracted by increased viscosity of the gradient. The gradient can be varied to optimise resolution in the range in which you are interested. A 5–40% gradient provides a range of about 25–1000 kDa and is a good starting point. Once you have determined the region of interest then the gradient can be varied to optimise separation of your protein or complexes. This is critical as poor resolution leads to an inability to separate out complexes of similar size. There are several ways to prepare a glycerol gradient, from various commercially available gradient pouring equipment, to layering by hand. We use the Gradient Master system to prepare gradients simply and reproducibly.

3.2.1. Preparation of Glycerol Gradient Using Gradient Master System

1. Place a 13 × 51 mm polyallomer tube in the metal marker block, and mark tube using upper level.
2. Fill tube up to mark with K300 containing 5% glycerol and protease inhibitors.
3. Fill syringe with 40% glycerol and use filling syringe to place 40% glycerol at the bottom of the tube, carefully filling up to the mark once again (keeping the end of the syringe just below the interface between the 5% and 40% glycerol) so the whole tube is now full: 40% glycerol below the mark and 5% above it.

4. Carefully insert a black lid into the top of the tube, keeping the tube level, and allowing any air to escape through the small hole in the lid.
5. Select 'SW50 short glycerol gradient 5–40%' on gradient maker, place tubes in the tube holder, and run the program.
6. When ready to load the sample, remove lid carefully, and gently layer your sample (*see* **Note 11**) onto the top of the gradient.

3.2.2. Setting Up a Glycerol Gradient Centrifugation Run

1. Cool the AH641 rotor and the buckets in the cold room for ~30–60 min.
2. Chill ultracentrifuge to 4°C.
3. Load gradient, place carefully in bucket, screw down the lid so the numbers on the lid and bucket align, and place in rotor (always run all buckets even if some are empty). Spin at 6900 g for 14 h, 30 min at 4°C (*see* **Note 12**).

3.2.3. Glycerol Gradient Fractionation

Perform steps 1–3 in the cold room.

1. Label 25 Eppendorf tubes 1–25 and chill in freezer for a few minutes before use.
2. Carefully remove 200 μl from the top of the gradient using a P1000 with a cut tip, taking the sample from the centre of the gradient, not the side (a P200 can also be used) and place into tube 1.
3. Repeat until glycerol gradient is completely fractionated. If there is any left, collect it in a tube labelled 26 (*see* **Note 13**).
4. (Optional) TCA precipitate the samples (*see* **Section 3.2.4**) to concentrate before SDS-PAGE analysis.
5. Load 10–15 μl of each sample on to SDS-PAGE to analyse. (Alternatively you can freeze the samples until needed.)

3.2.4. TCA Precipitation

1. Add 35 μl of 100% TCA to each 200 μl fraction (or 175 μl for a 1 ml fraction) to give a final concentration of 15% TCA (v/v).
2. Vortex for 30 s.
3. Incubate for 30 min on ice.
4. Spin at 15.7 g in a bench-top centrifuge for 15 min at 4°C.
5. Remove supernatant (careful with pellet).
6. Add 500 μl acetone and vortex for 30 s.
7. Spin at 15.7 g in a bench-top centrifuge for 15 min at room temperature.
8. Remove supernatant and dry pellet until acetone cannot be smelt (*see* **Note 14**).
9. Resuspend in 1 × SDS Loading Buffer for SDS-PAGE analysis.

3.3. Size-Exclusion Chromatography

Size-exclusion chromatography involves introducing cell extract or purified protein to a size-exclusion column of suitable

resolution, and collecting fractions. The column matrix consists of many tiny beads with pores of varying sizes. Large complexes cannot explore as much of the matrix as smaller complexes, so make a shorter path between the beads to the bottom of the column, resulting in a small elution volume. In addition to molecular weight, the shape of the protein complex also affects the elution volume; elongated complexes cannot pass through as many pores as their globular counterparts, and therefore behave as a globular complex of larger molecular weight. Size-exclusion chromatography can be used to estimate the Stokes radius of proteins or protein complexes. The choice of column used should be based on the size of the proteins or complexes being investigated. Ideally, the working range will be in the middle of the elution range. A good resolution is essential for accurate measurements, therefore we strongly recommend the Sephacryl S-500 column (Amersham). This column gives good resolution over a wide range. The HPLC system used should be able to provide constant pressure, such as the Akta purification system.

Perform All Steps in Cold Room/Cold Cabinet.

1. Determine column void volume. This is the elution volume of the smallest particle that cannot fit through any of the pores in the column matrix. This can be determined by running a 1 mg/ml solution of Dextran blue (included with the molecular weight standards from Amersham) through the column, and measuring the elution volume either by SDS-PAGE analysis or measuring the absorbance of each fraction at 380 nm using a spectrophotometer. The void volume is the volume at which Dextran blue begins to elute.
2. Run the globular protein standards in the first run (*see* **Section 3.4.1**), and repeat at least twice under the conditions you will use for your protein or extract (*see* **Note 15**).
3. Wash the column with 2 column volumes (CV) water; then equilibrate with 1 CV K150 or K300 buffer (*see* **Note 16**).
4. Load sample (retain an aliquot to serve as an input lane for analysis). It is best to keep the sample volume as low as possible; 1–2% of CV is a good guide. Run the column at 0.1 ml/min overnight (*see* **Note 17**) collecting 1 ml fractions (*see* **Note 18**).
5. TCA precipitate if necessary (*see* **Section 3.2.4**) and analyse using SDS-PAGE.

3.4. Hydrodynamic Calculations

3.4.1. Calibration Curves

It is essential to calibrate the glycerol gradient and size-exclusion chromatography systems for the exact conditions used in your experimental setup. Make up a solution containing protein standards covering the MW range of interest (we use a mixture of

the Low and High molecular weight standards from Amersham). Weigh out 5 mg/ml (1 mg/ml for Ferritin) of each standard and dissolve in 1 ml water or K300/K150 buffer (+3% glycerol for use in glycerol gradients). Run 50 μl on a glycerol gradient and 0.5–1 ml on size-exclusion chromatography. At this concentration you should be able to analyse the fractions using SDS-PAGE without the need for TCA precipitation. Note that several of these standards are homomultimeric and so will separate into monomers of smaller molecular weight under denaturing SDS-PAGE conditions. Perform at least two independent standard runs for each technique. You will use the known Stokes radius and Svedberg coefficients for the standards to construct calibration curves.

Svedberg Coefficient (From Glycerol Gradient)
Plot a graph with the *x*-axis as Svedberg coefficient and the *y*-axis as the peak fraction from glycerol gradient centrifugation (use the mean from three independent runs). Add the data from the globular standards and plot a linear regression line through the points. Use the equation of the regression line to estimate the Svedberg coefficient of your complex of interest, again using the mean peak fraction from at least two independent runs.

AQ1

Stokes Radius (From Size-Exclusion Chromatography)
Plot a graph with the *x*-axis as the Stokes radius and the *y*-axis as –log K_{av}. The constant –log K_{av} is used because its relationship with the Stokes radius is linear, and is calculated from **Equation** (**5.1**):

$$K_{av} = \frac{\text{elution volume } (V_e) - \text{Void volume } (V_0)}{\text{Total volume } (V_t) - V_0} \qquad (5.1)$$

V_e = elution volume in ml.
V_0 = Void volume of the column.

Add the data from the standards run and plot a linear regression line through the points. Again, use the equation of the regression line to estimate the Stokes radius of your complex of interest from the experimentally determined K_{av}.

AQ2

3.4.2. Native Molecular Weight Determination

Once you have estimated the Svedberg coefficient and Stokes radius of the protein or complex of interest, the native molecular weight can be determined from **Equation** (**5.2**) (*see* **Note 19**).

$$MW(Da) = \text{Stokes radius (cm)} \times \text{Svedberg coefficient } (10^{-13}\,\text{s}) \times \frac{6\pi\eta_0 N}{(1 - \upsilon p)} \qquad (5.2)$$

η_0 = viscosity of H_2O ($\sim 1.005 \times 10^{-2}$ g/cm.s) *(4)*.
N = Avogadro's number (6.022×10^{23}/mol).

υ = partial specific volume ($\sim$ 0.724 cm^3/g, or can be more accurately determined using values for each amino acid *(5)*).
p = density of solvent ($\sim$ 0.998 g/cm^3).

3.4.3. Calculation of Axial Ratio

Once the Stokes radius and Svedberg coefficient have been determined and the native molecular weight calculated, you can calculate an axial ratio for your protein or complex, assuming a prolate ellipsoid shape. First calculate the frictional ratio, the ratio between the translational frictional coefficient (f) of the measured Stokes radius and the translational frictional coefficient (f_0) of a perfect sphere with the same molecular weight as your protein or complex, to give the ratio f/f_0:

$$(f/f_0) = \frac{\text{Stokes radius (cm)}}{[(3\upsilon\,\text{MW})/(4\pi\text{N})]^{1/3}} \tag{5.3}$$

MW = native molecular weight determined above (in Daltons), υ and N as before *(4)*.

A frictional ratio of about 1 indicates that your protein or complex is likely to be globular, whereas values above 1 indicate a nonglobular state.

To obtain the axial ratio, the Perrin or shape function P, can be used. P is related to $f/f_0$and the hydration value (ω) of the protein:

$$P = (f/f_0)[(\omega/\upsilon\text{p}) + 1]^{-1/3} \tag{5.4}$$

Where ω can be estimated to be 0.35–0.40 and υ and p are as above.

The calculated value for P can then be used to give an indication of the axial ratio (a:b) of your protein or complex, assuming a prolate ellipsoid (rodlike) shape. The relationship between P and axial ratio is given in **Table 5.1**. This estimation of axial ratio is usually sufficient to give a rough idea of the elongation of your protein or complex (see *(6)* for a method to more accurately determine the axial ratio from P).

3.4.4. Calculation of Standard Deviation

To obtain a reasonable estimate of the error, the Stokes radius and Svedberg coefficients should be determined experimentally at least three times, and the mean and standard deviation of these values carried into the molecular weight and axial ratio calculations. When multiplying or dividing two constants with independent errors, the following equation should be used:
For example:

$$Z = AB: \qquad (z/Z)^2 = (a/A)^2 + (\mathbf{b}/\mathbf{B})^2,$$

Table 5.1
Axial ratios for prolate ellipsoids for the hydrodynamic function P (from *(5)*)

P Value	Axial Ratio
1.0000	1.0
1.0009	1.1
1.0031	1.2
1.0063	1.3
1.0103	1.4
1.0149	1.5
1.0201	1.6
1.0256	1.7
1.0315	1.8
1.0377	1.9
1.0440	2.0
1.1130	3.0
1.1830	4.0
1.2500	5.0
1.3140	6.0
1.3750	7.0
1.4340	8.0
1.4900	9.0
1.5430	10
1.9960	20
2.3590	30
2.6710	40
2.9500	50
3.2050	60
3.4420	70
3.6640	80
3.8740	90
4.0740	100

Source: Reproduced from *(6)*.

where z is the standard deviation for Z, b is the standard deviation for B, and a is the standard deviation for A. Bear in mind this does not include additional error which is introduced by other assumptions made during the hydrodynamic analysis.

3.4.5. Example Using the NDC80 Complex

The NDC80 kinetochore complex is an evolutionarily conserved four-protein kinetochore complex that is essential for

kinetochore–microtubule attachment in yeast and humans *(7–9)*. This complex has been shown by EM and rotary EM to form an elongated rodlike structure *(10, 11)*. We use the NDC80 complex as an example to illustrate the use of hydrodynamic analysis in determining the native molecular weight and elongation of a stable kinetochore complex.

First, following the instructions in **Section 3.4.1**, size-exclusion chromatography and glycerol gradient systems are calibrated. Next, mitotically arrested HeLa cell extract is fractionated on size-exclusion chromatography and glycerol gradients and the fractions are analysed using antibodies to the Ndc80 subunit of NDC80 (**Fig. 5.1A,B**). This profile reveals one discrete peak (N1) indicating that Ndc80 is part of a single Ndc80-containing complex. The Stokes radius and Svedberg coefficient can then be calculated:

Stokes radius:
Elution volume = 82 ml (**Fig. 5.1A**).

$$K_{av} = \frac{(V_e) - (V_0)}{(V_t) - V_0} = \frac{(82–33)}{(120–33)} = 0.5632$$

Regression line from calibration curve:

$$y = 0.002x + 0.0504$$

($y = -\log\ K_{av}$, x = Stokes radius (Å))

$$\text{Therefore Stokes radius} = \frac{-\log 0.5632–0.0504}{0.002}$$

Stokes radius = 99.4Å

Repeat experiment three times, and obtain mean Stokes radius ± standard deviation:
Run 1: 82 ml
Run 2: 82 ml
Run 3: 84 ml
Mean Stokes radius = 94.67 ± 8.3 Å.

Svedberg coefficient:
Peak fraction = 8 (**Fig. 5.1D**).
Regression line from calibration curve:

$$y = 1.2608x + 1.9274$$

(y = peak fraction, x = Svedberg coefficient).

$$\text{Therefore the Svedberg coefficient} = \frac{8–1.9274}{1.2608}$$

Svedberg coefficient = 4.82

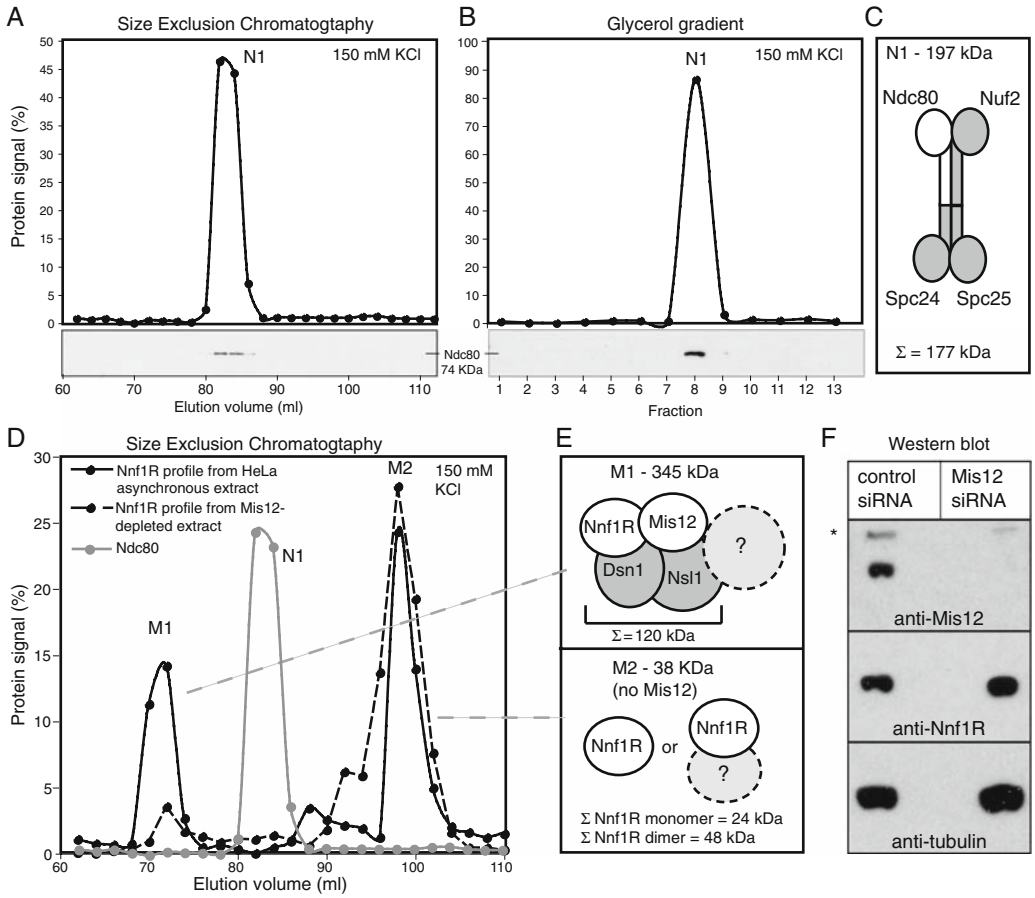

Fig. 5.1. Hydrodynamic analysis of the Ndc80 and MIND complexes from human cells. Whole cell extracts were prepared from nocodazole-arrested HeLa cells and fractionated on a HiPrep 16/60 Sephacryl S-500 HR size-exclusion chromatography column (**A**), and a 5–40% glycerol-density gradient (**B**), before immunoblotting with anti-Ndc80 antibodies *(13)*. (**C**) Cartoon of the Ndc80 complex indicating the predicted molecular weight calculated from the amino acid sequences of the subunits (Ndc80 = 74 kDa, Nuf2 = 54 kDa, Spc24 = 22.5 kDa, Spc25 = 26 kDa). (**D**) Whole cell extracts were prepared from asynchronous HeLa cells (solid black line) or from Mis12 siRNA-treated cells (dashed black line) and fractionated on a HiPrep 16/60 Sephacryl S-500 HR size-exclusion chromatography column before immunoblotting with antibodies to Nnf1R *(12)*. The profile of the NDC80 complex is overlaid in grey to illustrate that the NDC80 and MIND complexes can be clearly resolved using human mitotic cell extracts. (**E**) Cartoon representing the possible composition of the Nnf1R-containing complexes observed in (**D**) (Nnf1R = 24 kDa, Mis12 = 24 kDa, Dsn1 = 40 kDa, Nsl1 = 32 kDa). (**F**) Immunoblots of whole cell extracts from control and Mis12 siRNA *(15)* treated cells using antibodies to Mis12, Nnf1 *(12)*, and tubulin (Sigma) showing that Mis12 is efficiently depleted upon treatment with Mis12 siRNA whereas Nnf1R protein levels are unaffected (*represents a cross reacting band).

Repeat experiment three times, and obtain mean Svedberg coefficient ± standard deviation:
Run 1: fraction 8
Run 2: fraction 9
Run 3: fraction 8
Mean Svedberg coefficient = 5.08 ± 0.46 Å

Native Molecular weight:
From these experimentally determined values we can now calculate the native molecular weight of the Ndc80-containing complex using **Equation** (**5.2**):

$$\begin{aligned}\mathrm{MW(Da)} &= \text{Stokes radius (cm)} \times \text{Svedberg coefficient}(10^{-13}\,\mathrm{s}) \\ &\quad \times \frac{6\pi\eta_0 \mathrm{N}}{(1-\upsilon\mathrm{p})} \\ &= (94.67 \times 10^{-8}) \times (5.08 \times 10^{-13}) \\ &\quad \times \frac{6\pi(1.005 \times 10^{-2})(6.022 \times 10^{23})}{(1-(0.724\text{–}0.998)} \\ &= 197897.7\,\mathrm{Da} \\ &= 197\,\mathrm{kDa}\end{aligned}$$

Standard deviation (s.d):

$$[\mathrm{s.d}/197]^2 = [8.3/94.67]^2 + [0.46/5.08]^2 = 24.8\,\mathrm{kDa}$$

Therefore the native molecular weight of the Ndc80 complex determined using hydrodynamic analysis from mitotically arrested HeLa cells is 197 ± 24.8 kDa, which is in good agreement with the theoretical molecular weight of the human NDC80 complex of 177 kDa.

Next, we can calculate the elongation of this complex using **Equations** (**5.3**) and **(5.4)**:

$$\begin{aligned}(f/f_0) &= \frac{\text{Stokes radius (cm)}}{[(3\upsilon\mathrm{MW})/(4\pi\mathrm{N})]^{1/3}} \\ &= \frac{96.5 \times 10^{-8}}{[(3 \times 0.724 \times 191000)/(4\pi \times 6.022 \times 10^{23})]^{1/3}} = 2.5\end{aligned}$$

Because the f/f_0 value is well above 1, we already know that this complex is distorted from a globular state. The Perrin, or shape function, *P*, can now be determined:

$$\begin{aligned}\mathrm{P} &= (f/f_0)[(\omega/\upsilon\mathrm{p}) + 1]^{-1/3} \\ &= 2.5[(0.375/0.724 \times 0.998) + 1]^{-1/3} = 2.17\end{aligned}$$

Table 5.1 relates a *P* value of 2.17 to an axial ratio of between 20:1 and 30:1 assuming a prolate ellipsoid shape.

This is in good agreement with structural studies of the human NDC80 complex *(10, 11)* and demonstrates the power of hydrodynamic analysis in determining the size and shape of multiprotein complexes from mammalian cell extracts.

3.5. siRNA as a Tool to Probe Multiprotein Complexes

The human MIND/Mis12 kinetochore complex contains four protein subunits; Nnf1R (Pmf1), Mis12, Nsl1, and Dsn1. Using antibodies to Nnf1R *(12)* and the approach described for the NDC80 complex we can analyse the hydrodynamic properties of the MIND complex. The MIND complex runs as two species on size-exclusion chromatography (**Fig. 5.1D**) and glycerol gradient ultracentrifugation *(13)* with molecular weights of ~38 kDa (M1) and ~345 kDa (M2). In order to investigate the composition of these complexes further, siRNA-mediated protein depletion can be used in combination with hydrodynamic analysis. Depletion of one subunit from a protein complex can result in the disintegration of the complex, degradation of the other components, or a shift in molecular weight of the complex. In the case of the MIND complex, depletion of the Mis12 subunit causes the degradation of the Dsn1 subunit, but not the Nnf1R subunit (*(14)*; **Fig. 5.1F**). (Currently the stability of the Nsl1 subunit in Mis12 depleted cells is unknown.) Therefore, as a simple example we can analyse the hydrodynamic properties of Nnf1R in asynchronous HeLa cell extracts depleted of Mis12 to determine whether the M1 and/or M2 MIND complexes contain Mis12 (*see* **Note 20**).

1. Design 3 siRNA oligos to target your gene (*see* **Note 21**). We used the following sequence to target Mis12: GGACAUUUUGAUAACCUUUTT *(15)*.
2. Validate the specificity and efficiency of the siRNA treatment using Western blotting or indirect immunofluorescence (*see* **Note 22**). **Figure 5.1F** shows the specific and efficient depletion of Mis12 in Mis12 siRNA but not control siRNA-treated cells.

DAY 1

1. Culture HeLa cells in a 10 cm dish until 80–90% confluency is reached.
2. Trypsinize cells: wash twice with PBS then add 1 ml 0.5% Trypsin-EDTA to the dish. Incubate at 37°C for 2–3 min until cells detach from the dish.
3. Take up HeLa cells in 10 ml fresh DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin.
4. Add 240 μl of cell suspension into 10 ml fresh DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin in another 10 cm dish

DAY 2

5. Change media to 6 ml MEM media supplemented with 10% FBS and 1% penicillin/streptomycin.
6. Mix the following components in Eppendorf tubes with filtered tips:
   Tube 1: 600 μl Optimem; 18 μl siRNA oligos (at 20 μM)
   Tube 2: 144 μl Optimem; 36 μl oligofectamine

7. Wait 8 min; then add tube 2 to tube 1 and mix by pipetting.
8. Wait 25 min;, then add the contents of tube 1 dropwise to cells.

DAY 3

9. Change media to fresh DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin.

DAY 4

10. After an appropriate time for efficient depletion of your protein add nocodazole to the cells (*see* **Note 23**).
11. After 14 h nocodazole treatment, harvest cells and prepare mitotic extract as in **Section 3.1**.
12. Follow instructions in **Sections 3.2** and **3.3** to determine the hydrodynamic properties of your complex of interest in the absence of your chosen subunit.

The size-exclusion elution profiles of Mis12-depleted extract and control extract can be seen in **Fig. 5.1D**. The larger molecular weight species (M2) is completely abolished when Mis12 is depleted, whereas the lower molecular weight species (M1) is unaffected. This indicates that Mis12 is normally present in the M2 complex but not the M1 complex. Furthermore, because we know that Dsn1 is degraded in the absence of Mis12 *(14)*, we can conclude that Dsn1 is also not part of the smaller molecular weight complex. The possible compositions of this 38 kDa (M1) complex therefore include a monomer of Nnf1R, a dimer of Nnf1R, a heterodimer of Nnf1 and Nsl1, or a heterodimer of Nnf1 and an alternative protein. Follow-on experiments using depletion of the other MIND subunits will lead to a more complete understanding of the composition of these two species. Combining siRNA with hydrodynamic analysis in this way is therefore a powerful approach to dissect the components of multiprotein complexes from human cells.

4. Notes

1. It is best for the lysis buffer (H100 here) not to contain detergent as this may disrupt native complexes, however, your protein of interest may be insoluble (present in low-speed or high-speed pellet; check these if you cannot detect your protein in the supernatant) and may require the addition of small molecules or detergent to enhance solubility.
2. Either NaCl or KCl can be used.
3. This may vary for different cell lines, so test to see what achieves best arrest, aiming for 60–70% arrested (rounded-up) cells.

4. Use at least 150 mM NaCl or KCl to minimise nonspecific interactions between proteins and the column matrix.
5. Change Optimem bottle every month and close bottle well as pH changes over time.
6. We have used HeLa cells in the main for these experiments. Alternative human cell lines can also be used, and we have also used HEK293 cells with equal success, however because HEK293 cells do not attach very strongly to the base of the dish, mitotic shake-off cannot be performed inasmuch as most of the interphase cells also detach during this process. Mitotic enrichment using nocodazole can still be performed, however.
7. We find that 8–10 15 cm dishes give about 1 ml of 30 mg/ml extract.
8. To stop here drip the suspended cells into Falcon tubes containing a little liquid nitrogen, allow nitrogen to boil away completely, and then place in –80°C freezer. The samples can be stored at –80°C for several weeks if necessary.
9. It is worth testing to see if three rounds give a better yield, although we found no difference between two and three rounds.
10. Remember, the extract currently contains 100 mM KCl from the lysis buffer. We use a final extract concentration of 150 mM for most of our analyses because this is close to physiological salt concentration. However, you may want to consider increasing the salt to a maximum of 600 mM if your complex of interest appears to be aggregating in high molecular weight complexes, in order to investigate lower-order assemblies. With high salt extracts, we advise also increasing the salt concentration in the glycerol gradient and size-exclusion chromatography running buffers.
11. The sample must be in less than 60% of the lowest percentage of glycerol used in the gradient to avoid the sample sinking into the gradient before centrifugation, and up to 200 μl total volume.
12. Select the soft spin and brake off settings on the centrifuge if available, because this will minimise any perturbation of the gradient during acceleration and deceleration.
13. It is useful to know if anything is aggregating or very large and therefore reaching the bottom of the gradient.
14. Be careful not to overdry the pellet as this makes it difficult to redissolve the pellet in SDS loading buffer.
15. It is also good to periodically run a set of standards to check that the calibration is still accurate.
16. We do not usually add protease inhibitor cocktail to this buffer due to the large volumes required, but protease inhibitors could be added if protein degradation is a problem.

17. The column can be run faster, but this slower speed gives the best resolution, which is important when distinguishing between complexes of similar sizes.
18. Once the void volume has been determined there is no need to collect any fractions before the void volume has been eluted.
19. Note that this method only gives an estimate of MW and has a typical systematic error of about ±20% of the calculated value.
20. Depletion of Mis12 abrogates the spindle checkpoint so it is not possible to prepare mitotically arrested extract in this case.
21. You will need to use a program like BLOCK-iT™ siRNA Designer on the Invitrogen website to find an appropriate sequence. If this is the first time you are investigating a gene it is best to order three siRNA oligos and test them all.
22. It is best to determine the efficiency of protein depletion using indirect immunofluorescence or Western blotting if antibodies are available to your protein. If antibodies are not available, reverse transcriptase PCR can be used as a measure of the siRNA efficiency. Test 24, 48, 72, and 96 h transfections and different concentrations of siRNA to determine the most suitable time and least amount for efficient protein depletion.
23. It is sometimes not possible to obtain mitotically arrested cells when depleting certain kinetochore proteins because in many cases their depletion abrogates the mitotic checkpoint. In this case, skip the addition of nocodazole and simply harvest cells at the end of the RNAi treatment to make an asynchronous extract.

Acknowledgments

The authors would like to thank the members of the McAinsh lab for helpful discussions and reading of the manuscript. We would also like to thank Hiro Yamano for equipment and expertise relating to the glycerol gradient ultracentrifugation experiments. This work was supported by Marie Curie Cancer Care.

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