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Sedimentation Velocity Analytical Ultracentrifugation. Validation of a simple cell-alignment protocol

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Abstract

In establishing the sources of data variability within SV-AUC analysis and their relative importance recent studies have demonstrated that alignment of the sample cells to the centre of rotation is the most significant contributing factor to overall variability. In this study we demonstrate the usefulness of a simple manual alignment protocol with the aid of x10 magnifying eye-piece and show it is possible to approach the performance of more sophisticated mechanical alignment tools.

Using a 0.7mg/ml bovine serum albumin solution a range of off-set angles were investigated and compared. The effect of increased mis-alignment angle is shown to cause an increased peak broadening for both the BSA monomer and dimer peaks whereas only the dimer shifts to lower sedimentation coefficients. Reducing the misalignment to within ±1 degree of true alignment minimizes these effects and we show this is possible using both our manual alignment protocol and commercially available manual alignment procedures.

Background

In establishing the sources of data variability within SV-AUC analysis and their relative importance Arthur et al (2009) demonstrated that alignment of the sample cells to the centre of rotation is the most significant contributing factor to overall variability. Working with a known irreversible monomer/dimer antibody mixture containing 3% dimer they showed the dimer peak to broaden and migrate to smaller sedimentation coefficients with increased angle of mis-alignment when analysed by the *SedFit* algorithm of P. Schuck (Schuck, 2000). The monomer showed a similar broadening however the sedimentation coefficients was unaffected. A similar picture was shown by Gabrielson and Arthur (2011) for a reversible bovine serum albimin (BSA) monomer/dimer system. A schematic of the AUC cell 'centrepiece off-set' relative to the axis of rotation is given in Figure 1.

Experimental Design

In this study we evaluate the Nanolytics Instruments GmbH mechanical alignment tool to set true alignment (0^o off-set), Figure 2, and employed an in-house 'mis-alignment tool', Figure 3, to set a range of off-set angles to confirm the above broadening effect and visualise the associated cell index mark off-sets, Figure 4.

A solution of bovine serum albumin at a concentration of 0.7mg/ml in phosphate chloride buffer (pH =6.8, I=0.10) was used. Samples were run in a four port rotor using cells with 12mm double sector centrepieces at 45k rpm for 5hrs at 20^oC under a sedimentation velocity protocol using absorbance and interference optics. After sedimentation the cells were removed from the rotor and re-dispersed using a roller mixer protocol for 20 mins, ready for the next alignment angle run. The samples were prepared in PBS buffer (pH = 6.8, I=0.1M) which was also used for the AUC reference (reference sector volume= 405µl, sample volume= 395µl). To validate the 're-dispersion protocol' the 0^o off-set sample was run in triplicate, two consecutive runs and a third at the end of mis-alignment series. A fourth run using a '0^o manually aligned' cell, with only the cell and rotor indexing marks for alignment, was also conducted as a direct comparison against the Nanolytics tool. In addition the following mis-alignment angles where

investigated, 0^o (x3), $+1^o$, $+2^o$, $+4^o$, -1^o, -2^o, -4^o and analysed using a c(s) model within the *SedFit* program (Schuck et al 2000). Only the BSA data is reported here.

Results and Discussion

The *SedFit* analysis of the BSA absorbance data, employing a friction ratio f/f_0=1.25 in the c(s) model, showed two primary peaks in good agreement with the expected molecular weight for the monomer and dimer of BSA with low residuals (typical rmsd=0.005, Figure 5). The effect of increased mis-alignment angle is clearly shown to cause an increased peak broadening for both the monomer and dimer whereas only the dimer shifts to lower sedimentation coefficients (Figure 6), which is consistent with the studies of Gabrielson and Arthur (2009, 2011). From these data, peak height was selected as a good indication of peak broadening and was used to calculate a Coefficient of Variation for the three 0^o off-set samples and shown to be 2.5%. Within this variation, is the variation due to the solute re-dispersal protocol which can be concluded to be minimal and thus shown to be an effective means of re-dispersal. Plotting peak height against mis-alignment angle shows a consistent height reduction through increments in both +ve and –ve values of off-set angle in a symmetrical form (Figure 6). The symmetry of this plot suggests that the centrepiece is correctly aligned to the cell casing via the centrepiece notch and that the 0^o setting is a 'true alignment' (Figures 1 and 4). Finally in comparing the 0^o samples aligned using the Nanolytics mechanical alignment tool with our 'very best' 0^o manual alignment using only the cell and rotor indexing marks, with the aid of a x10 magnifying eye piece, we observed the peak height to be within the 2.5% error calculated above. These data would suggest that if we align the AUC cells to within $\pm 1^o$ using a manual alignment with a x10 magnifying eye-piece it is possible to approach the performance of the mechanical alignment tool.

The data from this trial also poses a question about the mass transfer mechanisms which operates within the cell sector due to mis-alignment where 'cross plane transfer' may have a role to play

(Figure 1). A key determinant here is the width of the optical detection window about the centre of the centrepiece sector. Additionally, consideration must be given to the computational handling of such data and the possibility of artefact formation within *SedFit* (Schuck, 2000). Certainly it is worth contrasting the different monomer and dimer shifts patterns in the context of the respective boundary velocities/profiles for the monomer and dimer due to the passage of the dimer through a 'monomer' solution against the passage of the monomer pure solvent (ie. the 'sedimented dimer phase').

Concluding Remarks

From this study we can confirm earlier observations that cell alignment has a significant effect on the broadening of peaks when processed using a c(s) model in *SedFit*. For multicomponent systems this can lead to peak overlap which can compromise the analysis. The data in this study would suggest that if we align the AUC cells to within $\pm$ 1^{o} using a manual alignment with the aid of x10 magnifying eye-piece it is possible to approach the performance of the alignment tool. Beyond this level of alignment precision, other limitations in the instrumentation such as temperature control and accuracy of data capture can become more significant limitations . As the accuracy in these areas also improves (Zhao et al, 2014), new sophisticated optically based alignment procedures (Doyle et al, 2017) will then become increasingly significant.

Acknowledgments

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Figure 1. Schematic diagram of the AUC cell 'centrepiece off-set' relative to the axis of rotation

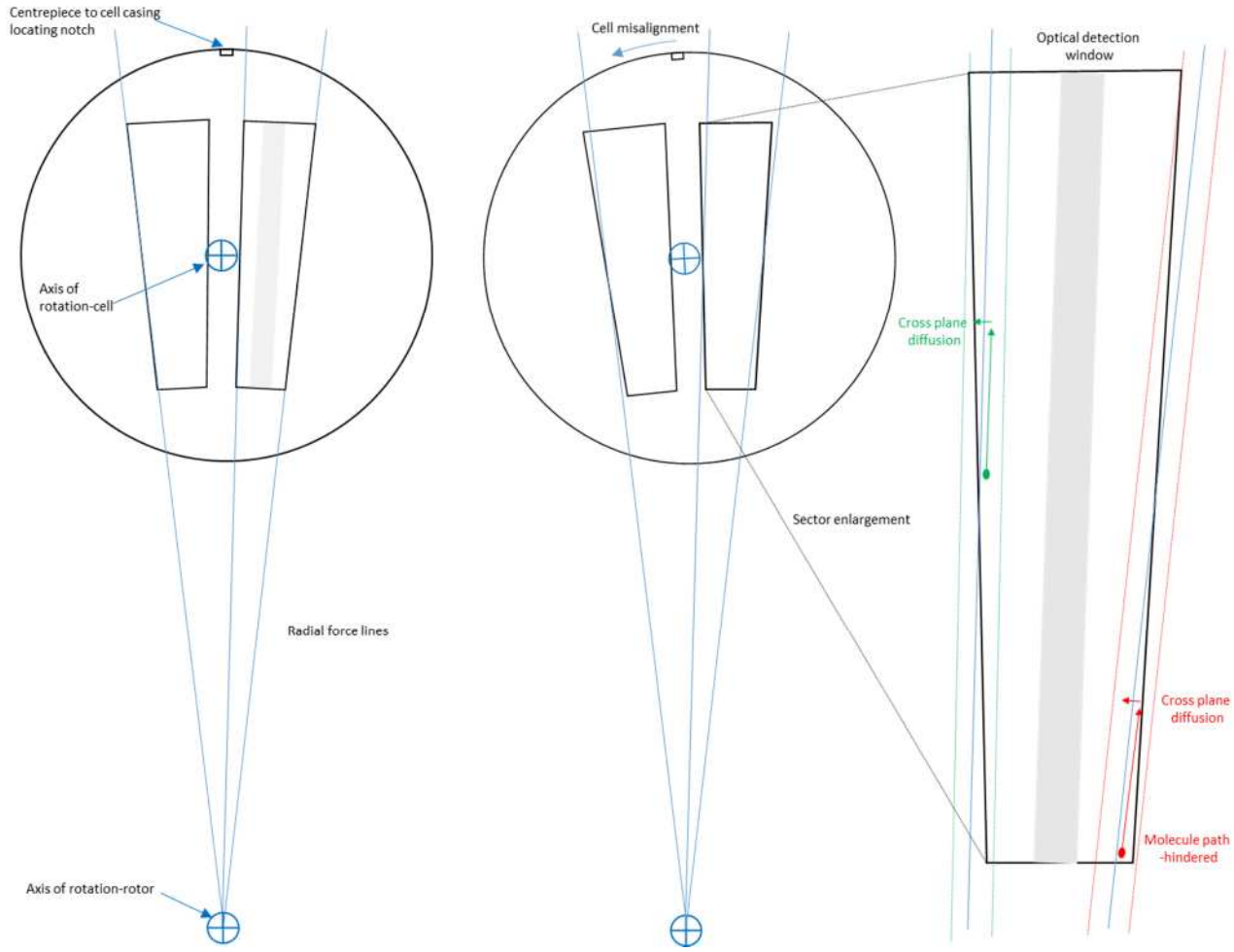

Figure 2. Nanolytics Instruments GmbH alignment tool (Courtesy of Dr. Kristian Schilling)

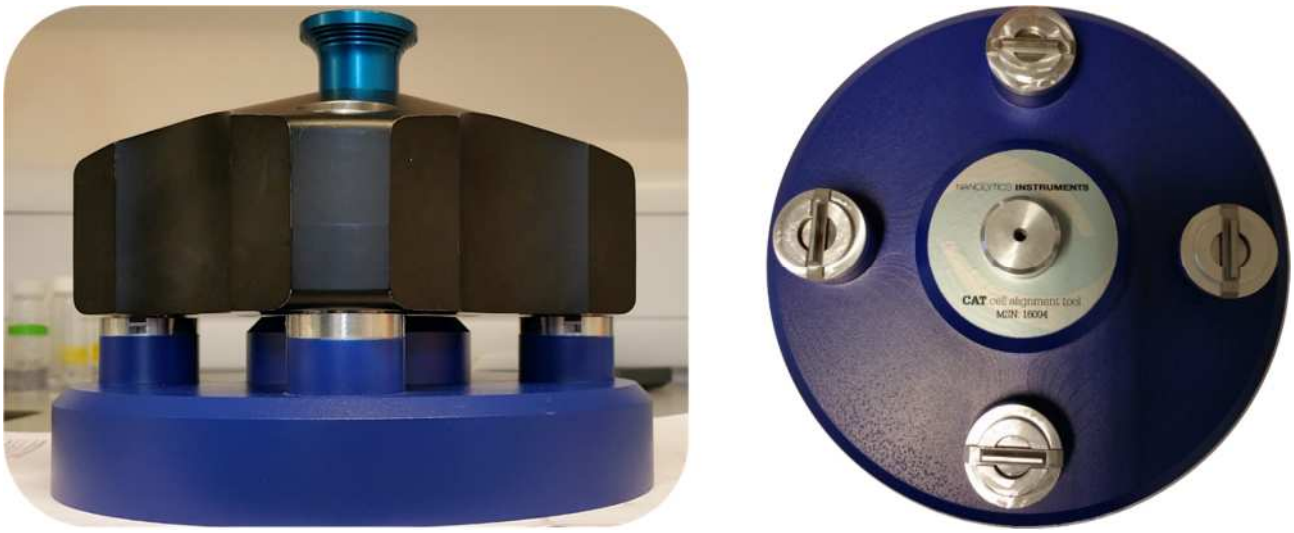

Figure 3. In-house 'mis-alignment tool'

Figure 4. Cell index mark off-sets as a function of mis-alignment angle

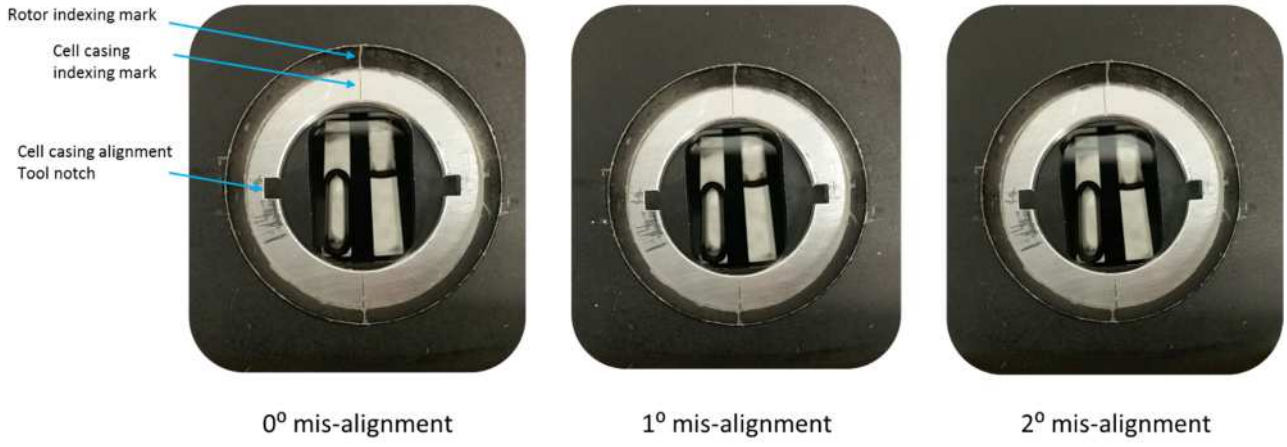

Figure 5. *SedFit* analysis of BSA absorbance data using a c(s) model, with a frictional ratio (f/f_o) =1.25

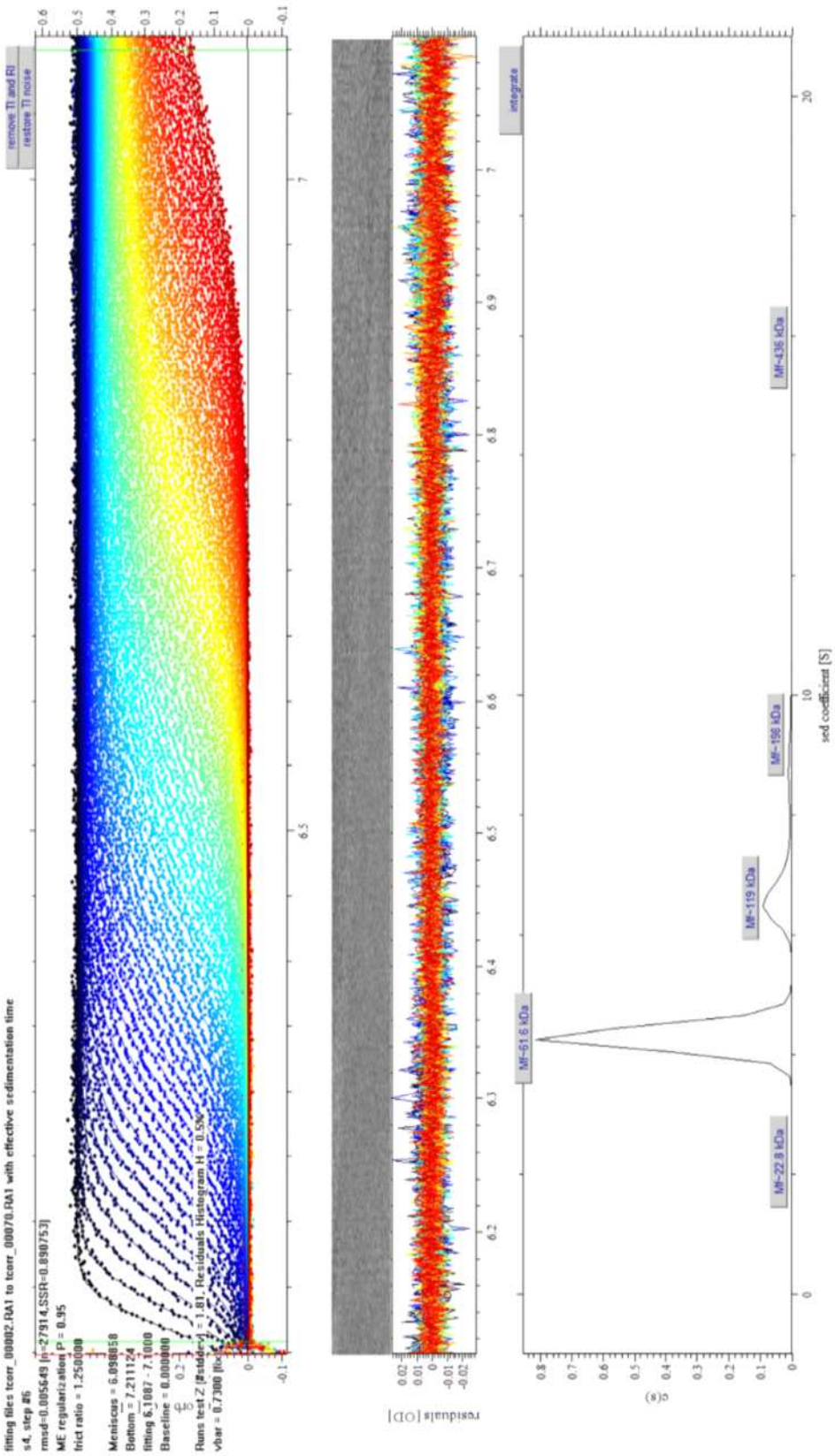

Figure 6. Sedimentation coefficient distribution plot for 0.7mg/ml bovine serum albumin in phosphate chloride buffer (pH 6.8, I=0.1) at a range of mis-alignment angles. Insert: Monomer peak height as a function of mis-alignment angle