Characterization of Single Proteins Using Double Nanohole Optical Tweezers

by

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B.Sc., Ben-Gurion University, 2012

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MASTER OF APPLIED SCIENCE

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University of Victoria

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Abstract

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Proteomic studies at the single molecular level could provide better understanding of the protein’s behaviour and the affects of its interactions with other biomolecules. This could have an impact on drug development methods, disease diagnosis, and targeted therapy. Aperture assisted optical trapping is a proven technique for isolating single proteins in solution without the use of tethers or labels, and without denaturing them. Thus enabling studies of protein-protein interactions, protein-small molecule interactions, and protein-DNA interactions.

In this work, double nanohole (DNH) optical tweezers were used to analyze the protein composition of heterogeneous mixtures. The trapped proteins were grouped by molecular mass based on two metrics: standard deviation (SD) of the trapping laser intensity fluctuations, and the time constant (τ) of the autocorrelation function of these fluctuations. The quantitative analysis is demonstrated first for two separate standard-size proteins, then for a mixed solution of both. Finally, the approach is applied to real unprocessed egg white solution. The results correspond well with the known protein composition of egg white found in the literature.

The DNH optical tweezers’ ability to distinguish proteins in unpurified heterogeneous mixtures, can progress this technique to the next level, allowing for single biomolecular studies of unprocessed physiological solutions like blood, urine, or saliva.
# Table of Contents

Supervisory Committee .............................................................................................. ii
Abstract .......................................................................................................................... iii
Table of Contents ........................................................................................................... iv
List of Tables .................................................................................................................. vi
List of Figures ................................................................................................................. vii
Acknowledgments .......................................................................................................... xiv
Dedication ....................................................................................................................... xv
Glossary .......................................................................................................................... xvi

Chapter 1 - Introduction ............................................................................................... 1
  1.1 Different Techniques for Protein Analysis ................................................................. 1
  1.2 Double Nanohole Optical Tweezers for Single-Molecule Analysis ......................... 3
  1.3 Motivation for This Thesis ....................................................................................... 3
  1.4 Organization of this Thesis ..................................................................................... 4
  1.5 Author’s Contribution ............................................................................................ 4

Chapter 2 - Background and Theory ........................................................................... 6
  2.1 Rayleigh Scattering and Conventional Optical Tweezers ........................................ 6
  2.2 Aperture Assisted Optical Tweezers ..................................................................... 11
    2.2.1 Bethe’s Aperture Theory and Self-Induced Back-Action .................................. 11
    2.2.2 Double Nanohole Optical Trapping ................................................................. 14
  2.3 DNH Optical Tweezers Application for Single Biomolecule Studies .................... 16
  2.4 Summary ............................................................................................................... 25

Chapter 3 - Experimental Methods ............................................................................. 26
  3.1 Nanoaperture Fabrication ....................................................................................... 26
    3.1.1 The FIB Machine ............................................................................................ 28
    3.1.2 DNH Fabrication ........................................................................................... 30
    3.1.3 Fiduciary Marker Fabrication ....................................................................... 32
    3.1.4 Challenges in Working with the Nanoapertures ............................................ 33
  3.2 Biological Solution Preparation and Sample Handling ......................................... 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 Trapping Setup</td>
<td>37</td>
</tr>
<tr>
<td>3.4 Data Collection and Analysis</td>
<td>39</td>
</tr>
<tr>
<td>3.5 Summary</td>
<td>44</td>
</tr>
<tr>
<td>Chapter 4 - Results and Discussion</td>
<td>45</td>
</tr>
<tr>
<td>4.1 Trapping of Size Standard Proteins</td>
<td>46</td>
</tr>
<tr>
<td>4.2 Trapping of a Mixed Solution</td>
<td>48</td>
</tr>
<tr>
<td>4.3 Egg White Analysis</td>
<td>49</td>
</tr>
<tr>
<td>4.4 Summary</td>
<td>60</td>
</tr>
<tr>
<td>Chapter 5 - Summary and Future Work</td>
<td>61</td>
</tr>
<tr>
<td>5.1 Conclusions</td>
<td>61</td>
</tr>
<tr>
<td>5.2 Future Work</td>
<td>62</td>
</tr>
<tr>
<td>Bibliography</td>
<td>63</td>
</tr>
<tr>
<td>Appendix - Matlab Code</td>
<td>72</td>
</tr>
</tbody>
</table>
List of Tables

Table 1: Main proteins known to be found in egg white $^{89,91}$ ............................................. 50

Table 2: Classification of egg white protein composition. Left: Grouping of experimental data (as shown in Figure 32), and classification by molecular weight ($M_r$). Right: Main proteins found in egg white, grouping by molecular weight, and scaled abundance in egg white. Reprinted with permission from$^{35}$ © 2018, American Chemical Society................................................................. 52
List of Figures

Figure 1  Scattering force and gradient force acting on a dielectric sphere displaced from the axis of a Gaussian laser beam. The wavelength of the beam is much smaller than the size of the sphere. (a) The curved lines at the left and right represent the shape of the laser beam and the Gaussian curve represents the intensity profile of the beam. “a” and “b” represent a typical pair of light rays striking the sphere symmetrically about its centre. The refraction of light by the particle changes the momentum of the photons, which result in the forces $F_a$ and $F_b$. These forces are resolved in two components: $F_{\text{scat}}$ pointing in the direction of the optical axis and $F_{\text{grad}}$ pointing towards the beam’s waist. (b) A strongly-focused Gaussian laser beam creates a dominant backward axial gradient force over the forward-scattering force. This results in a stable three-dimensional trap. Reprinted with permission from $^4$ © 2000, IEEE................................................................. 8

Figure 2  Optical transmission through a subwavelength aperture. (a) Red-shifting of the transmission curve caused by a dielectric particle, leading to an increase in transmission ($\Delta T$). (b) No particle in the aperture. (c) Transmission is enhanced due to a dielectric particle in the aperture. (d) Transmission is decreased by $\Delta T$ as the particle tries to escape the aperture. The total photon momentum through the aperture decreases, which induces the force $F$ that pulls the particle back into the aperture. Reprinted with permission from $^5$ © 2013, Journal of Visualized Experiments................................................................. 14

Figure 3  Double-nanohole (DNH) trapping aperture. (a) Schematic view of the DNH structure in a metal film. (b) Scanning electron microscope (SEM) image of a DNH with $T=100$ nm, $D=178$ nm, $W=50$ nm, and $L=205$ nm. (d) Finite-difference time-domain (FDTD) simulated field intensity distribution in the DNH with an excitation wavelength of 828 nm. Reprinted with permission from $^6$ © 2015 Optical Society of America. (c) Experimental and FDTD simulation transmission spectra of a DNH aperture, showing a peak at 805 nm. Reprinted with permission from $^7$ © 2014, American Chemical Society ........................................................................................................................................ 16
Figure 4  Time traces of the optical power transmitted through the double-nanohole (DNH). Showing the reversibility of trapping by turning the laser on and off, using an incident optical power of (a) 13.4 mW and (b) 5.3 mW. Reprinted with permission from\textsuperscript{32} © 2012 American Chemical Society ................................................................. 17

Figure 5  Time traces of the trapping signal of bovine serum albumin (BSA). Using an incident optical power of (a) 13.4 mW [(b) zoom-in of (a)], (c) 10.6 mW, and (d) 8.5 mW. The vacant state and two trapping states (T1 and T2) are clearly shown. Reprinted with permission from\textsuperscript{32} © 2012 American Chemical Society ................................................................. 18

Figure 6  Co-trapping of BSA with anti-BSA. (a) BSA is flowing through the microfluidic channel. (b) A single BSA particle is trapped between the tips of the double-nanohole. (c) Anti-BSA is then flowing in. (d) Anti-BSA is bound to the BSA molecule and both are co-trapped. Reprinted with permission from\textsuperscript{65} © 2013 The Royal Society of Chemistry ................................................................. 19

Figure 7  (a) Demonstration of single protein binding using the double nanohole aperture: (i) flowing 20 nm biotin-coated polystyrene particles, (ii) trapping event of 20 nm biotin-coated polystyrene particle in the double nanohole aperture and subsequently flowing streptavidin, (iii) binding of streptavidin with the trapped biotin-coated polystyrene particle. (b) First control experiment: (i) flowing 20 nm biotin-coated polystyrene, (ii) trapping event of 20 nm biotin-coated polystyrene particle and subsequently flowing saturated streptavidin, (iii) saturated streptavidin does not bind to the trapped 20 nm biotin-coated polystyrene particle. (c) Second control experiment: (i) flowing 20 nm non-functionalized polystyrene particles, (ii) trapping event of 20 nm polystyrene particle and then flowing streptavidin, (iii) streptavidin does not bind to the trapped 20 nm polystyrene particle. Reprinted with permission from\textsuperscript{66} © 2013 Optical Society of America ........................................................................................................ 20

Figure 8  Autocorrelation functions of trapped signals for streptavidin with (red) and without (blue) biotin. Reprinted with permission from\textsuperscript{68} © 2014 American Chemical Society ........................................................................................................ 21
Figure 9  Trapping and unzipping of DNA, with and without the tumor suppressor protein p53. (a) Single strand DNA trapping event with no intermediate step. (b) A hairpin DNA trapping event showing the unzipping with an intermediate step of \( \sim 0.1 \) sec. (c) The wild type p53 suppresses the unzipping of the DNA hairpin for a delay of \( \sim 5 \) sec. (d) The mutant p53 is incapable of suppressing the unzipping of the DNA hairpin. Reprinted with permission from\(^71\) © 2014 Optical Society of America .......................................................... 23

Figure 10  Raman spectra of single proteins. (a) Twenty-two different sweeps across 11 trapping events of carbonic anhydrase, showing a singular broad peak centred around 38 GHz. (b) Twenty different sweeps across 10 trapping events of ovotransferrin, showing two distinct peaks and a single finely split peak. Red curves in (a) and (b) show the average of all sweeps. (c) A single frequency sweep for the blood protein cyclooxygenase-2, showing two peaks at 95 GHz and 120 GHz. (d) A single frequency sweep for aprotinin, showing two peaks around 40 GHz. Reprinted with permission from\(^76\) © 2014 Springer Nature ........................................................................................................... 24

Figure 11  Raman spectra of ssDNA fragments. (a) Beating frequency sweep for a 20 base ssDNA showing a single peak around 40 GHz. (b) Beating frequency sweep for a 20 base ssDNA showing a fundamental resonant frequency at 28.3 Hz and a second order harmonic at 57.6 GHz. Reprinted with permission from\(^77\) © 2015 the Royal Society of Chemistry ........................................................................................................... 25

Figure 12  Scanning electron microscope (SEM) image of the gold film adhered to the glass slide ........................................................................................................... 27

Figure 13  Gold samples ready to go into the FIB machine. Two samples can be mounted on the stage holder at the same time. This allows for fabricating two different samples with the same process of aligning the machine’s beams. The fiduciary scratch is seen in the middle of the left sample, pointing to the ROI for fabrication .............................................. 27

Figure 14  Schematic of a focused ion beam system with a liquid-metal ion source. Reprinted with permission from\(^78\) © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim .................................................................................................................. 28
Figure 15  FB-2100 FIB machine software interface for configuring milling parameters.

Figure 16  SEM images of DNH aperture fabricated with the FIB machine. (a) Top view. (b) 52° angle tilt.

Figure 17  DNHs fabricated in a gold sample. SEM image at magnification of 1715x. 9 DNHs are marked with red arrows.

Figure 18  SEM image of the fabricated sample at magnification of 500x. The end of the scratch (red circle) can be seen on the left and the squared fiduciary marker is on the right. In the centre (blue circle) a smaller marker can be detected. This marker indicates a specific distance from the centre DNH aperture. The DNHs are too small to be seen at this magnification.

Figure 19  SEM images of newly milled DNH apertures, fabricated using a single beam Hitachi FB-2100. Both apertures were milled in the same run, 50 microns apart under the same settings but have noticeably different gap sizes of approximately 20 nm...

Figure 20  SEM images of newly milled DNH apertures, fabricated using a dual beam FEI Helios NanoLab 650. Both apertures were milled in the same run, 150 microns apart under the same settings and have only a slight difference in gap sizes. Reprinted with permission from© 2018, American Chemical Society.

Figure 21  SEM images of a DNH aperture after continuous trapping of a week. (a) Top view. (b) 52° angle tilt. The apertures seem to lose sharpness around the cusps and edges. Reprinted with permission from© 2018, American Chemical Society.

Figure 22  Sample preparation procedure. (a) The gold sample (DNHs facing up), adhesive spacer, biological solution, no. 0 glass slide, tweezers. (b) The adhesive spacer is mounted in the centre of the glass slide. (c) The solution is pipetted into the micro-well formed by the spacer. (d) The gold sample is mounted on top of the solution. DNHs are facing down, immersed in the solution.
Figure 23  Schematic of the aperture-assisted trapping setup. Containing: a laser diode; half wave plate (HWP); polarizer; mirror (MR); beam expander (BE); dichroic mirror (DM); optical density filter (ODF); avalanche photodiode (APD); data acquisition card (DAC). Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society.... 37

Figure 24  Local intensity enhancement (linear scale) for a DNH of 25 nm gap and 190 nm diameter excited at 633 nm with a linear polarization parallel to the apex between the holes. Reprinted with permission from\textsuperscript{82} © 2015, Springer Nature........................ 38

Figure 25  Trapping event of an egg white protein. Blue curve shows the raw data, sampled at 100 kHz. Red curve shows the data downsampled to a frequency of 1 kHz. (a) The untrapped state and the trapped one are indicated. Trapping is identified from a sudden increase in transmission. (b) Zoom in on the untrapped signal. (c) Zoom in on the trapped signal.............................................................. 39

Figure 26  Autocorrelation function for a trapping event of an egg white protein. ACF of the untrapped (blue) and trapped (red) states with a two-exponent function fit to ACF of the trapped signal (dashed black). Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society .............................................................. 42

Figure 27  Examples of disregarded data, while trapping egg white proteins. (a) The signal looks noisy as soon as the laser power is back on, probably due to a molecule stack close to the trap. The signal increases gradually, then stabilizes and looks like a normal trapping event. (b) A second jump in the laser transmission after the first trapping event. We assume this is a result of a second molecule entering the trap. Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society ............................................ 43

Figure 28  RMS variation of trapped particles, autocorrelation of trapped particles. (a) RMS of the trapped particles with respect to their molecular weight. (b) Autocorrelation relaxation time for the trapped particles with respect to their molecular weight. Reprinted with permission from\textsuperscript{83} © 2015, the Royal Society of Chemistry................................................. 46
Figure 29  Histograms of trapping data. Bimodal distribution of voltage values for ovotransferrin. Reprinted with permission from\textsuperscript{83} © 2015, the Royal Society of Chemistry ................................................................. 47

Figure 30  Standard deviation (SD) and autocorrelation function time constant ($\tau$) of ovotransferrin and ovalbumin trapping events. (a) Pure solutions of ovotransferrin (black triangles) and ovalbumin (blue circles). (b) Mean ± 1 standard deviation of ovotransferrin (black) and ovalbumin (blue) SD results. The two data points were fit to $y = 0.05x - 0.74$ (red).......................................................................................................................... 48

Figure 31  Standard deviation (SD) and autocorrelation function time constant ($\tau$) of ovotransferrin and ovalbumin trapping events. (a) A mixed solution of ovotransferrin and ovalbumin (1:1 ratio by volume). Group A was classified as ovotransferrin particles; group B was classified as ovalbumin particles. (b) Mean ± 1 standard deviation of group A (black) and group B (blue) SD results. The two data points were fitted to $y = 0.05x - 0.81$ (red). Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society.................... 49

Figure 32  Standard deviation (SD) and autocorrelation function time constant ($\tau$) of egg white trapping events. Trapping events in group A (black) were classified as particles with a molecular weight higher than 49 kDa; trapping events in group B (blue) were classified as trapped particles with a molecular weight in the range 36–49 kDa; trapping events in group C (green) were classified as trapped particles with a molecular weight lower than 39 kDa. (a) SD vs. $\tau$ plot. (b) log(SD) vs. log($\tau$) plot. The distribution is fit to $y = -0.634x - 1.59$ (red). Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society .................................................................................. 51

Figure 33  SEM image of the nanoaperture (DNH) used for trapping egg white proteins............................................................................................................................... 52

Figure 34  Trapping events of pure ovotransferrin molecules. (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.19e - t 4.69 + 0.01e - t 1380$ (red). ............... 54
Figure 35  **Trapping events of pure egg white protein molecules from Group A** (Figure 32). (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.35e^{-t2.8} + 0.03e^{-t153}$ (red). (b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.19e^{-t3.91} + 0.06e^{-t164}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from© 2018, American Chemical Society

Figure 36  **Trapping events of pure ovalbumin molecules.** (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.7e^{-t23.4} + 0.3e^{-t183}$ (red).

Figure 37  **Trapping events of pure egg white protein molecules from Group B** (Figure 32). (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.77e^{-t13.8} + 0.22e^{-t383}$ (red). (b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.79e^{-t9.87} + 0.29e^{-t71.3}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from© 2018, American Chemical Society

Figure 38  **Trapping events of pure ovomucoid molecules.** (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.7e^{-t23.36} + 0.3e^{-t186}$ (red).

Figure 39  **Trapping events of pure egg white protein molecules from Group C** (Figure 32). (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.58e^{-t27} + 0.33e^{-t320}$ (red). (b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.58e^{-t21.75} + 0.37e^{-t439}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from© 2018, American Chemical Society
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Dedication

To all international students at UVic trying to survive this hostile campus.

May you all graduate quickly, with a bit more dignity and sanity than I have left in me.
## Glossary

### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACF</td>
<td>Autocorrelation Function</td>
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<tr>
<td>APD</td>
<td>Avalanche Photodiode</td>
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<tr>
<td>BE</td>
<td>Beam Expander</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
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<tr>
<td>DAC</td>
<td>Data Acquisition Card</td>
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<tr>
<td>DNH</td>
<td>Double Nanohole</td>
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<tr>
<td>EAR</td>
<td>Extraordinary Acoustic Raman</td>
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<td>FDTD</td>
<td>Finite-Difference Time-Domain</td>
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<tr>
<td>FIB</td>
<td>Focused-Ion beam</td>
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<td>HWP</td>
<td>Half Wave Plate</td>
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<tr>
<td>LED</td>
<td>Light-Emitting Diode</td>
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<tr>
<td>LMIS</td>
<td>Liquid-Metal Ion Source</td>
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<tr>
<td>NA</td>
<td>Numerical Aperture</td>
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<tr>
<td>NAFT</td>
<td>Nanoaperture Fiber Tweezer</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>ODF</td>
<td>Optical Density Filter</td>
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<tr>
<td>OIMO</td>
<td>Oil Immersion Microscope Objective</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PSMI</td>
<td>Protein Small Molecule Interaction</td>
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<td>RMS</td>
<td>Root Mean Square</td>
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<td>ROI</td>
<td>Region of Interest</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>SIBA</td>
<td>Self-Induced Back-Action</td>
</tr>
</tbody>
</table>
List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>Gold</td>
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<tr>
<td>c</td>
<td>Speed of Light</td>
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<td>E</td>
<td>Electric Field</td>
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<td>Ga</td>
<td>Gallium</td>
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<td>H</td>
<td>Magnetic Field</td>
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<td>Planck Constant</td>
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<td>I</td>
<td>Laser Intensity</td>
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<tr>
<td>k_B</td>
<td>Boltzmann Constant</td>
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<tr>
<td>M_r</td>
<td>Molecular Mass</td>
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<tr>
<td>n</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>p</td>
<td>Dipole Moment</td>
</tr>
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<td>R</td>
<td>Autocorrelation</td>
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<td>r</td>
<td>Radius</td>
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<td>T</td>
<td>Temperature</td>
</tr>
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<td>Ti</td>
<td>Titanium</td>
</tr>
<tr>
<td>U</td>
<td>Potential Energy</td>
</tr>
<tr>
<td>Z_o</td>
<td>Free Space Impedance</td>
</tr>
<tr>
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<td>Polarizability</td>
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<td>γ</td>
<td>Stokes’ Drag Coefficient</td>
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<td>η</td>
<td>Viscosity</td>
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<td>Trap Stiffness</td>
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<td>Wavelength</td>
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<td>ξ</td>
<td>White Noise</td>
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<tr>
<td>σ²</td>
<td>Variance</td>
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<tr>
<td>τ</td>
<td>Time Constant</td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction

Proteins are remarkable molecules which are the most versatile and essential functional elements of life\(^1\). They are the fundamental components responsible for nearly all biological processes, overseeing cell development and catalyzing metabolic reactions\(^2\). The protein is also one of the four principle building blocks of the cell, the smallest unit of our body\(^3\). Given their important function, defects in proteins can result in enzyme deficiency which leads to mutations and diseases\(^4,5\). Thus, the study of proteomics emerged to understand protein function and their interactions\(^6\), in addition to the development of technologies to detect, quantify, and analyze these molecules\(^7–9\).

Protein biomarkers play a significant role in disease diagnostics. In 2012, the US National Institutes of Health (NIH) recognized the need to develop new technologies for improving the ability to identify and quantify proteins in complex samples: “our ability to identify and quantify proteins in complex (e.g., clinical) samples is progressing steadily, but it is clear that orders-of-magnitude improvements in the associated technologies would enable substantial advances in a large range of biomedical research areas”\(^9\).

Proteins also interact with other proteins and small molecules to initiate different processes and control the function of cells in our body. Proteins can change their location within the cell and depending on their function, change their shape as they bind to other molecules, store, and release them\(^10\). Dynamic and three-dimensional studies of proteins provide information on their conformational changes and interaction kinetics.

1.1 Different Techniques for Protein Analysis

Two well established methods can be used for characterizing the proteins in heterogeneous mixtures: gel electrophoresis\(^11\), and mass spectrometry\(^12\). Targeted proteomics mass spectrometry is one of the leading methodologies for detecting and analysing specific proteins, but it is also an expensive technology and cannot be accessed in high demand\(^9,13\). Both electrophoresis and mass spectrometry require taking the proteins out of solution and denaturing them as part of a preparation process. Therefore the dynamics of the protein in
its natural environment cannot be studied using these traditional methods. Some of these preparation procedures require digestion of the protein sample into peptide products, subjecting it to sodium dodecyl sulfate polyacrylamide (SDS), and gas-phase ionization.

Some techniques such as fluorescence and dyeing methods, can be used for labelling and investigating in-solution biomolecular interactions and protein folding. A significant drawback of the chemical modification required for the labelling of the molecule, is that it affects the physical properties of the protein and alters its natural behavior. Other techniques such as nuclear magnetic resonance (NMR), can also study the structure and dynamic motion, but in an indirect and expensive way and often with the need of purified and concentrated solutions.

More recent developments have given rise to nanopore technologies that can be used to characterize the size and conformational changes of single proteins. Nanopore analytics uses a single electrolyte-filled pore in a thin insulating membrane that connects two solutions and serves as a channel for individual molecules to pass through. An electrical field is applied using two electrodes connected to these two solutions. When a molecule flows through the channel it temporarily distorts the electric field and blocks the ionic current through the pore. The frequency of these modulations can be used to infer the number of particles, while the magnitude of the modulations is proportional to their size. Due to the proteins’ small globular shape, this method has a hard time reducing their translocation time for detection without the use of tethering or electrophoretic solutions. Another drawback of this technique is that while detection of the molecule of interest can be achieved using nanopores, the isolation of these proteins for detailed studies has not yet been achieved.
1.2 Double Nanohole Optical Tweezers for Single-Molecule Analysis

Double nanohole (DNH) optical trapping, the technique used in this work, will be discussed at length in this thesis. The tweezers have been proven to be able to trap single molecules of proteins and nanoparticles\textsuperscript{27-32} while allowing the manipulation of the trapped particle and probing its kinetic behaviour\textsuperscript{33}. This method does not denature the protein and allows for dynamic studies at the molecular level. It has shown signatures for protein-protein interactions\textsuperscript{31}, protein-small molecule interactions\textsuperscript{34} and protein-DNA interactions\textsuperscript{33}. This detection platform is inexpensive and highly sensitive, and can provide aid to researchers in understanding of the biomolecular interactions, towards single-molecule based drug development.

1.3 Motivation for This Thesis

Most studies done using the DNH optical tweezers were done in homogenous solutions. Taking this technology to the next level will require showing its ability to isolate, identify and study proteins out of heterogeneous solutions as well. The work presented in this thesis is the first step for achieving this goal.

As a proof of concept, the protein content of egg white was analyzed by characterizing its trapping signals. This is the first time these tweezers were used with an unprocessed biological solution, and the results can lead the way for working with other physiological solutions, such as blood or urine samples.
1.4 Organization of this Thesis

This thesis first presents the theory behind aperture assisted optical trapping and describes some of its applications. Then the experimental methods used in this work are detailed and the results acquired are presented and discussed.

Chapter 2 covers the theoretical background of conventional optical tweezers and its expansion into the double nanohole (DNH) tweezers used in this work. It also details the progress made using this technique for biomolecule studies.

Chapter 3 lays out the steps in the process of running the experiment that were used in this work, including the fabrication procedure, materials and equipment used and the data analysis approach.

Chapter 4 presents the results obtained when studying size standard proteins and egg white proteins. It also discusses the results, along with a comparison to the known protein composition of egg white from the literature.

Chapter 5 concludes this work and suggests some future work.

1.5 Author’s Contribution

The work presented in Section “Chapter 4 - Results and Discussion” was published in a scientific journal:


We use a double nanohole optical tweezer to analyze the protein composition of egg white through analysis of many individual protein trapping events. The proteins are grouped by mass based on two metrics: standard deviation of the trapping laser intensity fluctuations from the protein diffusion, and the time constant of these fluctuations coming from the autocorrelation. Quantitative analysis is demonstrated for artificial samples, and then the approach is applied to real egg white. The composition found from real egg white corresponds well to past reports using gel electrophoresis. This approach differs from past works by allowing for individual
protein analysis in heterogeneous solutions without the need for denaturing, labeling or tethering.

This work was also presented in two different conferences and two conference papers were published:


The protein distribution of egg white is analyzed by single molecule light scattering in a double nanohole optical tweezer configuration. Different proteins are binned by their characteristic scattering profile.


Nanohole optical trapping is a tool that has been shown to analyze proteins at the single molecule level using pure samples. The next step is to detect and study single molecules with dirty samples. We demonstrate that using our double nanohole optical tweezing configuration, single particles in an egg white solution can be classified when trapped. Different sized molecules provide different signal variations in their trapped state, allowing the proteins to be statistically characterized. Root mean squared variation and trap stiffness are methods used on trapped signals to distinguish between the different proteins. This method to isolate and determine single molecules in heterogeneous samples provides huge potential to become a reliable tool for use within biomedical and scientific communities.
Chapter 2 - Background and Theory

This chapter covers the theory behind optical tweezers and specifically aperture assisted optical tweezers. It also reviews the progress made in our group with protein single molecule analysis using this technique. Optical trapping uses a focused laser beam to manipulate small objects, and it is used here to isolate and characterize biomolecules.

Section “2.1 Rayleigh Scattering and Conventional Optical Tweezers” presents the basic principles of optical trapping and its dynamics for the ray optics models and for Rayleigh particles. Section “2.2 Aperture Assisted Optical Tweezers” describes the technique used in this work. This technique relies on Bethe’s theory of transmission through small holes, and allows trapping of small molecules using low optical power, such that it is not damaging to them.

Finally, Section “2.3 DNH Optical Tweezers Application for Single Biomolecule Studies”, details the various works and results obtained by our group in the past. These studies are the basis as well as the motivation for the work presented in this thesis, aiming to enable using optical tweezers for studying biomolecules in heterogeneous mixtures.

2.1 Rayleigh Scattering and Conventional Optical Tweezers

The first optical tweezers were developed by Arthur Ashkin in the 1970’s\(^38,39\). Optical trapping has since been used in many applications of manipulating, assembling and sensing of micro-scale and nano-scale objects\(^40-42\). Optical tweezers use a strongly focused beam of light to trap objects. Intensity gradients in the converging beam draw small objects toward the focal point, while the radiation pressure of the beam pushes them up the optical axis. Under conditions where the gradient force dominates, a particle can be trapped, in three dimensions, near the focal point. Two optical forces are playing a role in optical tweezers, and the balance between them is what allows a particle to get trapped: the scattering force, resulting from the momentum transfer of photons hitting the particle; and the gradient force, resulting from the intensity gradient.
Figure 1 illustrates the radiation pressure acting on a particle in an electromagnetic field and the resulting scattering and gradient forces exerted on it. The particle here is much bigger than the wavelength of the beam, and has a higher refractive index than its surrounding medium. When a photon scatters off of the particle, it carries a linear momentum of:

$$ P = \frac{h \nu}{c} $$

Where $h$ is Planck constant, $\nu$ is the photon’s frequency, and $c$ is the speed of light.

A change in the direction of the beam’s propagation requires a force to be acting on the particle in the opposite direction, such that momentum is conserved. For a Gaussian beam, the lateral component of this reaction force is always pointing towards the centre, where the laser intensity is highest. Ray “a” in Figure 1a has a higher intensity than ray “b”, therefore $\|\vec{F_a}\| > \|\vec{F_b}\|$. Adding all these symmetrical pairs of rays striking the particle, the net force can be resolved into two components: The scattering force component ($F_{\text{scat}}$), pointing in the direction of the incident light; and the gradient force component ($F_{\text{grad}}$), arising from the gradient in light intensity and pointing transversely toward the high intensity region of the beam.

The dynamics of the system is similar to a ball and spring system. The gradient force pulls or pushes the particle depending on its location relative to the focal point. When the particle is on the optical axis, the sum of lateral forces acting on it is zero: $\vec{F}_{\text{grad}_x} = \vec{F}_{\text{grad}_y} = 0$. Thus the particle can be accelerated by the scattering force along the propagation direction of the beam. The stable three-dimensional trap forms by strongly focusing the beam, with a high numerical aperture (NA) microscope objective. The backward axial gradient force then becomes dominant and cancels out the forward scattering force, as shown in Figure 1b.
Figure 1  Scattering force and gradient force acting on a dielectric sphere displaced from the axis of a Gaussian laser beam. The wavelength of the beam is much smaller than the size of the sphere. (a) The curved lines at the left and right represent the shape of the laser beam and the Gaussian curve represents the intensity profile of the beam. “a” and “b” represent a typical pair of light rays striking the sphere symmetrically about its centre. The refraction of light by the particle changes the momentum of the photons, which result in the forces $F_a$ and $F_b$. These forces are resolved in two components: $F_{scat}$ pointing in the direction of the optical axis and $F_{grad}$ pointing towards the beam’s waist. (b) A strongly-focused Gaussian laser beam creates a dominant backward axial gradient force over the forward-scattering force. This results in a stable three-dimensional trap. Reprinted with permission from $^{43}$ © 2000, IEEE

The forces acting on a dielectric sphere in the ray optics regime (where the diameter of the sphere is greater than the wavelength of the incident light) can be calculated quantitatively using Fresnel reflection and transmission coefficients$^{44}$:

$$F_{scat} = \frac{nP}{c} \left\{ 1 + R \cos(2\theta) - \frac{R^2(\cos(2\theta - 2r) + R \cos(2\theta))}{1 + R^2 + 2R \cos(2r)} \right\}$$ (2)

$$F_{grad} = \frac{nP}{c} \left\{ R \cos(2\theta) - \frac{R^2(\sin(2\theta - 2r) + R \sin(2\theta))}{1 + R^2 + 2R \cos(2r)} \right\}$$ (3)

Where $n$ is the refractive index of the medium, $P$ is the power of the laser beam, $\theta$ is the angle of incidence, $r$ is the angle of reflection, and $R$ and $T$ are Fresnel reflection and transmission coefficients of the surface at $\theta$. 

In this work we are dealing with Rayleigh particles. Rayleigh scattering is a domain of electromagnetic theory that describes the interaction of light with subwavelength particles. In this case, the simple ray optics modeling that was used for illustration in Figure 1 cannot sufficiently describe the system’s behaviour. Here we use Maxwell’s equations in their time-independent form.

Consider a small sphere which is illuminated by a collimated beam of monochromatic, linearly polarized light. If the sphere is small enough, like in the case of a Rayleigh particle, the electric field surrounding it is approximately uniform across its volume. That induces a dipole in the particle, making it oscillate synchronously and in the same direction as the incident field. It is this oscillation dipole that radiates the electromagnetic energy and constitutes the scattering. The dipole moment is given as:

\[ \vec{p} = \alpha \vec{E} \]  

(4)

Where \( \vec{E} \) is the electric field and \( \alpha \) is the particle’s polarizability, which is proportional to the third power of its radius \( (r) \):

\[ \alpha = 4\pi r^3 (n_{med})^2 \left( \frac{n_{part}}{n_{med}} \right)^2 - 1 \left( \frac{n_{part}}{n_{med}} \right)^2 + 2 \]  

(5)

Where \( n_{med} \) is the refractive index of the surrounding medium and \( n_{part} \) is the refractive index of the particle.

The beam intensity if defined as:

\[ I = \frac{n_{med}c}{2} |E|^2 \]  

(6)

The optical power \( P_{scat} \) scattered by the particle can be approximated by the radiation of an electric dipole as:

\[ P_{scat} = \frac{16\pi^4 c}{3 \lambda^4} |\vec{p}|^2 \]  

(7)

Where \( \lambda \) is the wavelength of the incident light.

As a result of this scattered power, the flux pattern of electromagnetic radiation changes. The particle moves in the propagation direction of the laser beam, due to momentum transfer, while the force exerted on it is the scattering force:

\[ F_{scat} = \frac{n_{med}c}{e} P_{scat} \]  

(8)
By plugging Equations (4) through (7) into Equation (8), we can express the scattering force of the tweezers as:

\[ F_{\text{scat}} = \frac{128\pi\varepsilon_0^6 n_{\text{med}}}{3c\lambda^4} \left( \frac{n_{\text{part}}}{n_{\text{med}}} \right)^2 \left( \frac{n_{\text{part}}}{n_{\text{med}}} \right)^{2-1} \right] \frac{1}{2} I_0 \]

(9)

Where \( I_0 \) is the intensity at the incident position.

When inspecting Equation (9) it can be observed that the scattering pressure (force per unit area) is proportional to the light intensity and also to \( \left( \frac{r}{\lambda} \right)^4 \). As the particle size gets smaller than the wavelength of the beam, the scattering pressure decreases drastically.

The second optical force acting on the particle, is the force resulting from the gradient of the electric field intensity. This force is the Lorentz force, acting on the dipole induced by the electromagnetic field\(^{42,45}\):

\[ F = (p \cdot \nabla)E + \frac{1}{c} \frac{dp}{dt} \times B \]

(10)

By using the expression of the dipole \( p \) in Equation (4), we get:

\[ F = \alpha (E \cdot \nabla)E + \frac{\alpha E}{c} \times B \]

(11)

Using the identity:

\[ (E \cdot \nabla)E = \frac{1}{2} \nabla E^2 - E \times (\nabla \times E) \]

(12)

And the Maxwell-Faraday equation:

\[ \nabla \times E = -\frac{1}{c} \frac{\partial B}{\partial t} \]

(13)

We get:

\[ F = \alpha \left( \frac{1}{2} (\nabla E^2) + \frac{1}{c} \frac{\partial}{\partial t} (E \times B) \right) \]

(14)

\( E \times B \) is the beam intensity, \( I_0 \), which is time-constant in optical tweezers. Therefore the gradient force of the tweezers can be expressed as:

\[ F_{\text{grad}} = \frac{1}{2} \alpha \nabla E^2 \]

(15)

The polarizability, \( \alpha \) (Equation 5), is influenced by the effective refractive index: \( \frac{n_{\text{part}}}{n_{\text{med}}} \). For the case of a dielectric particle with a higher refractive index relative to that of the surrounding medium, the gradient force attracts the particle towards the beam’s waist, which is where the field intensity is highest. The polarizability, and sequentially the
gradient force, is also proportional to the third power of the particle’s radius. Notice that the scattering force is proportional to the sixth power of the radius (Equation 9). It is clear that as the particle gets smaller, the gradient force becomes more dominant.

2.2 Aperture Assisted Optical Tweezers

Small particles suspended in fluid experience more random motion (Brownian motion), compared to large ones\(^{50}\). This results in high thermal kinetic energy of the particles. In order to create a stable optical trap, the potential well of the trapping force should be significantly stronger than the kinetic energy of the particle. The potential energy \(U\) should then be\(^{51,52}\):

\[
U \gg k_B T
\]

Where \(k_B\) is Boltzmann constant, and \(T\) is the temperature.

The trapping potential for a dielectric sphere in an electromagnetic field can be expressed as\(^{53}\):

\[
U = -p \cdot E = \frac{2\pi n_{med} r^3}{c} \left(\frac{n_{part}}{n_{med}}\right)^2 - l_0 \left(\frac{n_{part}}{n_{med}}\right)^3
\]

One of the limitations for using optical tweezers for trapping small particles, lies in the third power dependence of the trapping potential on the particle’s radius (Equation 17). When the particle gets one order of magnitude smaller, the beam intensity must be increased by three orders of magnitudes. Therefore, stable trapping of Rayleigh particles using conventional optical tweezers requires higher power, which can have damaging effects on the particle. The use of high intensity laser is not suitable for biological molecules (biomolecules), which are very temperature sensitive. Therefore, different strategies need to be utilized for trapping these nanometer biological molecules.

2.2.1 Bethe’s Aperture Theory and Self-Induced Back-Action

The concept of aperture assisted optical tweezers is based on Bethe’s theory of diffraction by small holes. In 1944, Hans Bethe studied the diffraction of light in a sub-wavelength
aperture in an infinite plane\textsuperscript{54}. When light attempts to propagate through an aperture that is much smaller than the light’s wavelength, the light is being cut-off at the edges. The propagating wave cannot satisfy the boundary condition of the electric field being zero at the edges of the aperture, therefore the light is diffracted.

Bethe’s work used a quasi-static approximation of Maxwell’s equations. In this approximation the system is a plane wave incident on a circular aperture with a diameter much shorter than the wavelength, in an infinitely thin perfect electric conductor film. The plane wave incident normally to the film and the electric and magnetic fields are parallel to the film. The light transmitted through the circular aperture is approximated by the emission of a magnetic dipole.

The optical transmission, in free-space, is half of the total power radiated by this dipole and is expressed as\textsuperscript{47}:

\[ T = \frac{128\pi^3 n^4 r_a^6}{27\lambda_0^4} |H_0|^2 \]  \hspace{1cm} (18)

Where \( Z_0 \) is the impedance of free space, \( r_a \) is the aperture’s radius, \( H_0 \) is the magnetic field of the incident light, \( \lambda_0 \) is the wavelength in free-space, and \( n \) is the refractive index of the surrounding medium.

The axioms assumed in Bethe’s theory are obviously unrealistic. However, this theoretical approximation was extended to metallic films of finite thickness at visible frequencies where the perfect electrical conductor and infinitely thin approximations become invalid. The experimental observations show a qualitative agreement with the theoretical conclusions\textsuperscript{55}. Thus we expect the optical transmission through a subwavelength aperture in a real metal film, to behave similarly to Bethe’s prediction.

Normalizing the transmission to the area of the circular aperture (\( A = \pi r^2 \)), shows that the transmission through the aperture is dependent on the fourth power of the ratio between the radius of the aperture and the wavelength of the light. This causes a rapid decrease in transmission as the aperture’s size is scaled below the wavelength:

\[ \frac{T}{A} \propto \left( \frac{r}{\lambda} \right)^4 \]  \hspace{1cm} (19)
The wavelength of the light transmitted through the aperture is scaled with the refractive index $\lambda = \frac{\lambda_0}{n}$. An increase in the refractive index can result in a significant increase in the transmission:

$$T \propto \frac{1}{\lambda^4} \propto \frac{n^4}{\lambda_0^4}$$  \hspace{1cm} (20)

This ‘red’-shift of the transmission spectrum can be seen in Figure 2a.

Figure 2b shows the optical transmission through the aperture, which is mainly a diffraction of the light, and has a low power. Figure 2c illustrates a scenario where a particle enters the trap. The particle’s refractive index is higher than that of the medium inside the aperture (which is usually water). This effect is called a dielectric loading, where the optical transmission is increased significantly due to only the change in refractive index within the same aperture (Equation 20). Figure 2d describes the case of the particle attempting to get away from the aperture and ‘escape’ the trap. The transmission through the aperture in this case decreases, as a result of the change in the refractive index. Therefore, the net momentum of the light changes, which leads to an equal change in momentum in the opposite direction, as Newton’s third law states. This results in an optical force towards the aperture, effectively pulling the particle back into the trap. This phenomenon is called self-induced back-action (SIBA) trapping.\textsuperscript{56}
2.2.2 Double Nanohole Optical Trapping

SIBA based optical trapping uses a nanoaperture at the trapping site (where the laser beam is focused). The trapping aperture is used for enhancing the electric field of the trapping laser, confining the trapped particle to a well-defined small region and enabling the detection of trapping (by the sudden increase in the transmitted signal). Although circular apertures in metallic films were used at first to stably trap spheres smaller than 100 nm with low optical power, new aperture designs were needed for trapping smaller particles. Different geometries were designed for the nanoaperture, such as rectangular and bowtie apertures. Some of these nanoapertures have shown the ability to trap particles down to...
a size of 20 nm. The work in this thesis was done using a double-nanohole (DNH) aperture that is used in the majority of trapping based experiments performed by the nanoplasmonics group at UVic. The DNH aperture has shown the ability to trap particles down to the size of 1 nm, including single proteins and DNA strands of few base pairs, as discussed in Section “2.3 DNH Optical Tweezers Application for Single Biomolecule Studies”.

The geometry of the DNH is defined by the thickness of the metal- T, the diameter of the circular apertures- D, the distance between the two circular apertures- L (the centre-to-centre separation), the curvature of the cusps- C, and the width of the gap- W; as described in Figure 3a. The DNH is milled in a 100 nm gold film, as discussed in Section “3.1.2 DNH Fabrication”. A scanning electron microscope (SEM) image of a DNH is shown in Figure 3b, and a finite-difference time-domain (FDTD) simulation of its field intensity plotted in Figure 3d. The field intensity distribution shows an enhancement around the cusps of the aperture. An FDTD simulation was also performed for the spectrum of the transmission through the DNH, and is presented in Figure 3c along with experimental results. The main transmission peak here is at 805 nm, slightly shorter than the 850 nm trapping laser. It should be noted that the DNH aperture was found to have more than one resonance peaks, but in the work described in this thesis only one wavelength of trapping laser was used ($\lambda = 850 \text{nm}$).
Figure 3  Double-nanohole (DNH) trapping aperture. (a) Schematic view of the DNH structure in a metal film. (b) Scanning electron microscope (SEM) image of a DNH with T=100 nm, D=178 nm, W=50 nm, and L=205 nm. (d) Finite-difference time-domain (FDTD) simulated field intensity distribution in the DNH with an excitation wavelength of 828 nm. Reprinted with permission from© 2015 Optical Society of America. (c) Experimental and FDTD simulation transmission spectra of a DNH aperture, showing a peak at 805 nm. Reprinted with permission from© 2014, American Chemical Society

2.3 DNH Optical Tweezers Application for Single Biomolecule Studies

In aperture assisted tweezers, the light transmitted through the nanoaperture is being monitored, and a trapping event of a particle is detected by an abrupt increase in the transmission. Once trapped, the particle is held in place by the high intensity electric field created by the trapping laser beam within the nanoaperture. In addition to the ‘jump’ in transmission, a significant increase in the signal fluctuation occurs when the particle is trapped. This is attributed to the Brownian motion and the conformal changes of the trapped particle in the aperture. Valuable information about the trapped particle can be extracted from studying different characteristics of the transmission signal during the trapping state.
Different studies have implemented this method for exploring the dynamics of single molecules, interactions between different molecules, the number of the particles trapped and their sizes.

Optical tweezers based on the SIBA effect with a circular nanoaperture, were used to achieve stable trapping of 50 nm polystyrene particles, with a laser power of only 1 mW\textsuperscript{56}. This led the way for trapping sub 100 nanometers molecules, using low powers. Later, the technique was used for studying biomolecules. At first bovine serum albumin (BSA) molecules (M\textsubscript{r} =66.5 kDa) were trapped\textsuperscript{32}. In that first work, the wavelength of the laser was 820 nm and different optical powers were tried, varying from 3.5 mW to 13.4 mW, and the minimum power that was reported in achieving trapping was 5.3 mW. Figure 4 shows the release of the molecule after trapping. The laser beam is being turned off (technically it is being physically blocked from reaching the microscope objective). The transmission level drops back to zero and after ~10 seconds, when the trapping laser is reapplied, the transmission ‘jumps’ back to approximately the same level of amplitude.

![Figure 4](image_url)  
**Figure 4** Time traces of the optical power transmitted through the double-nanohole (DNH). Showing the reversibility of trapping by turning the laser on and off, using an incident optical power of (a) 13.4 mW and (b) 5.3 mW. Reprinted with permission from\textsuperscript{32} © 2012 American Chemical Society
Figure 5 shows the trapping signals of BSA using different optical powers. Three amplitude levels can be seen- The untrapped state (V=0, ‘vacant’), the first ‘jump’ of the signal (T1) which is assumed to be the trapping of the molecule and a second ‘jump’ (T2) which is attributed to the unfolding of the molecule between the N and F forms of BSA. Switching back and forth between the two trapping states (T1 and T2), shows reversible folding and unfolding of the protein. This indicates that the temperature at the trapping site does not rise above the denaturizing temperature of BSA, which is 50 °C.

Figure 5  Time traces of the trapping signal of bovine serum albumin (BSA). Using an incident optical power of (a) 13.4 mW [(b) zoom-in of (a)], (c) 10.6 mW, and (d) 8.5 mW. The vacant state and two trapping states (T1 and T2) are clearly shown. Reprinted with permission from© 2012 American Chemical Society

Label-free methods for single protein studies usually use binding of the protein to a surface for monitoring. This restricts the motion of the protein and interferes with its natural dynamics. Furthermore, by using some of the binding sites of the molecule for surface immobilization, part of the protein is physically blocked by the surface. Aperture assisted tweezers enable the detection of protein interactions in their native state, without tethering or any kind of immobilization.
The DNH trap was integrated with microfluidic channels, and was used to study the co-trapping of BSA and anti-BSA (Figure 6). First a stable trapping of a single BSA molecule was achieved, then anti-BSA was flowed into the channel where it was bound to the already trapped BSA molecule\textsuperscript{65}.

![Figure 6 Co-trapping of BSA with anti-BSA.](image)

(a) BSA is flowing through the microfluidic channel. (b) A single BSA particle is trapped between the tips of the double-nanohole. (c) Anti-BSA is then flowing in. (d) Anti-BSA is bound to the BSA molecule and both are co-trapped. Reprinted with permission from\textsuperscript{65} © 2013 The Royal Society of Chemistry

In another study, the same microfluidic system was used to detect the binding of streptavidin and biotin\textsuperscript{66}. Biotin, which is also known as vitamin B7 (or vitamin H) forms a non-covalent interaction with the protein called streptavidin (M\textsubscript{r} = 60 kDa). Streptavidin has an extraordinary high affinity for biotin, and this streptavidin-biotin complex has a dissociation constant on the order of 10\textsuperscript{-14} mol/L. In that work, biotin-coated polystyrene spheres were first trapped. Once a stable trapping was achieved, streptavidin was flown into the microfluidic channel. The binding of the streptavidin to the biotin was detected by a second sudden increase in the transmission (a jump), as can be seen in Figure 7a.

Two control experiments were conducted to confirm the second jump in transmission is indeed a result of the binding event. The first one is shown in Figure 7b, in which the binding sites of the streptavidin were blocked off (by mixing the streptavidin with excess
biotin before flowing it into the channel). No second jump was observed in that experiment. No second jump was observed also in the case where the polystyrene spheres that were first trapped were not coated with biotin. The streptavidin that was flown into the channel after the first trapping event, did not affect the transmitted signal (Figure 7c).

Figure 7  (a) Demonstration of single protein binding using the double nanohole aperture: (i) flowing 20 nm biotin-coated polystyrene particles, (ii) trapping event of 20 nm biotin-coated polystyrene particle in the double nanohole aperture and subsequently flowing streptavidin, (iii) binding of streptavidin with the trapped biotin-coated polystyrene particle. (b) First control experiment: (i) flowing 20 nm biotin-coated polystyrene, (ii) trapping event of 20 nm biotin-coated polystyrene particle and subsequently flowing saturated streptavidin, (iii) saturated streptavidin does not bind to the trapped 20 nm biotin-coated polystyrene particle. (c) Second control experiment: (i) flowing 20 nm non-functionalized polystyrene particles, (ii) trapping event of 20 nm polystyrene particle and then flowing streptavidin, (iii) streptavidin does not bind to the trapped 20 nm polystyrene particle. Reprinted with permission from© 2013 Optical Society of America
Protein-small molecule interactions (PSMIs) are fundamental to the protein’s function in living organisms. Studies have shown that a small molecule binding to the protein can substantially alter its molecular dynamics\(^67\). Studying these interactions can have a key role in drug development.

The DNH optical tweezers were used to observe real-time label-free free-solution single molecule dynamics of different complexes, showing significantly different behaviors between the protein with and without the small molecule binding\(^68\). When examining the biotin-streptavidin complex, time traces of the trapped signal of the bare form of streptavidin showed slower timescale dynamics as compared to the biotinylated form of the protein. Figure 8 shows the autocorrelation functions (ACF) of the trapped signals for the bare form of streptavidin, and for biotinylated streptavidin. The faster decaying time of the ACF of the biotinylated streptavidin implies that the bound form of the protein is subject to less conformal changes. This is consistent with other studies, which suggest that the four binding loops of the streptavidin molecule are highly mobile in the absent of biotin\(^69\). This method of analysis can be used for screening small molecule drug candidates by monitoring their influence on proteins of interest, and for understanding the mechanisms of PSMIs\(^70\).

Figure 8  Autocorrelation functions of trapped signals for streptavidin with (red) and without (blue) biotin. Reprinted with permission from\(^68\) © 2014 American Chemical Society
The DNH trap was also used for studying the unzipping of small hairpin DNA fragments, the interaction of a transcription protein with DNA, and its impact on the dynamics of the hairpin DNA\textsuperscript{71}.

A single-stranded DNA was first trapped and showed to have only one jump in transmission (Figure 9a). Then a hairpin DNA (10 base-pairs) was trapped and an intermediate transmission step was observed (Figure 9b). The initial change in transmission in that case is a result of the hairpin DNA molecule getting trapped, while the following increase indicates the unzipping of the molecule. The elongation due to unzipping, increases the polarizability of the molecule, resulting in a higher optical transmission. The typical time scale for unzipping a 10 base-pair hairpin DNA was $\Delta t = 0.1$ sec.

Next, the interaction between DNA and the tumor suppressor p53 was studied. Mutations of this tumor suppressor are implicated in approximately 75\% of known cancers in humans\textsuperscript{72}, which makes the study of its interactions with DNA of critical importance. Proteins binding to DNA can either stabilize or destabilize its structure and affect the unzipping behavior in the optical trap\textsuperscript{73}. Figure 9c shows the trapping signal of the p53 wild type protein-DNA complex with a longer unzipping time ($\Delta t$~5 sec). The increased unzipping time is associated with the strong binding of p53 to the DNA hairpin structure\textsuperscript{74}, which is critical for the biological activity of p53\textsuperscript{75}. To support the claim that this delay in the unzipping process is actually due to the binding of p53, the interaction of a mutant form of p53 with a hairpin DNA was examined as well. Figure 9d shows the trapping signal for p53 mutant-DNA complex. The intermediate step in this case is of the same time scale as for the case of trapping the hairpin DNA by itself.

These results show the DNH tweezers’ ability to study the dynamics of small DNA fragments and their interactions with different types of other proteins.
Figure 9  Trapping and unzipping of DNA, with and without the tumor suppressor protein p53. (a) Single strand DNA trapping event with no intermediate step. (b) A hairpin DNA trapping event showing the unzipping with an intermediate step of $\sim 0.1$ sec. (c) The wild type p53 suppresses the unzipping of the DNA hairpin for a delay of $\sim 5$ sec. (d) The mutant p53 is incapable of suppressing the unzipping of the DNA hairpin. Reprinted with permission from© 2014 Optical Society of America

The DNH trap was also used with extraordinary acoustic Raman (EAR), for exciting acoustic vibrational modes of single proteins. These experiments were based on the electrostriction force in the trap, created by the electric field, that pulls on the particle. Two tunable trapping lasers were used, instead of the single laser that is usually used for trapping. After trapping the protein the lasers were detuned from each other, creating a beat frequency between them. The beat frequency was changed to cover the range of 10-300 GHz. The beat frequency affects the electrostriction force on the trapped molecule. When it matches one of the acoustic modes of the protein, the level of fluctuations of the molecule in the trap is maximized. The fluctuations were measured by the root mean squared (RMS) variation of the transmission signal. The resonant frequency of each protein was detected
from its Raman spectrum, when plotting the RMS of the signal against the beat frequency\textsuperscript{76,77}. This approach showed a resolution of almost three orders of magnitude finer than conventional Raman spectroscopy. The results indicate the DNH tweezers’ ability to measure the size, shape and material properties of nanoparticles.

Figure 10 shows the Raman spectra of four different proteins: carbonic anhydrase, ovotransferrin, blood protein cyclooxygenase-2, and aprotinin.

![Raman spectra of single proteins](image)

**Figure 10** Raman spectra of single proteins. (a) Twenty-two different sweeps across 11 trapping events of carbonic anhydrase, showing a singular broad peak centred around 38 GHz. (b) Twenty different sweeps across 10 trapping events of ovotransferrin, showing two distinct peaks and a single finely split peak. Red curves in (a) and (b) show the average of all sweeps. (c) A single frequency sweep for the blood protein cyclooxygenase-2, showing two peaks at 95 GHz and 120 GHz. (d) A single frequency sweep for aprotinin, showing two peaks around 40 GHz. Reprinted with permission from\textsuperscript{76} © 2014 Springer Nature
The EAR experiment was also used to excite and detect the resonant vibrational modes of single stranded DNA (ssDNA) containing tens of bases. Figure 11a shows the Raman spectrum of a 20 base ssDNA, with a resonant vibration frequency of 40 GHz. Figure 11b shows the Raman spectrum of a 30 base ssDNA, showing a resonant frequency of 28.3 GHz.

To study the influence of the size of ssDNA on the resonant vibrational frequency, different lengths of ssDNA (20 to 40 bases) were measured. The measured frequencies showed good agreement with analytic 1-D lattice vibration theory. This shows the DNH tweezers’ ability to characterize very short DNA strands with a resolution of a few bases.\textsuperscript{77}

![Figure 11](image)

**Figure 11**  **Raman spectra of ssDNA fragments.** (a) Beating frequency sweep for a 20 base ssDNA showing a single peak around 40 GHz. (b) Beating frequency sweep for a 20 base ssDNA showing a fundamental resonant frequency at 28.3 Hz and a second order harmonic at 57.6 GHz. Reprinted with permission from\textsuperscript{77} © 2015 the Royal Society of Chemistry

\textbf{2.4 Summary}

The fundamental principles of the DNH optical tweezers were presented in this chapter, as well as the advantages and applications of this tool. The work presented in this thesis implements the same theory and methods with the aim of enabling future studies of single biomolecules dynamics, not only with clean purified solutions, rather straight out of heterogeneous (dirty) mixtures.
Chapter 3 - Experimental Methods

This chapter describes the methods used for experimenting with the aperture assisted optical tweezers. The trapping setup used for the experiment was built at the beginning of this work and is presented in Section “3.3 Trapping Setup”. Section “3.2 Biological Solution Preparation and Sample Handling” details the procedure for preparing the protein sample to go into the trapping setup before each experiment. Section “3.1 Nanoaperture Fabrication” describes the process of fabricating the nanoaperture (DNH) in the gold substrate. A new aperture must be fabricated in order for a new data set to be collected, and that is the first step for the experiment.

3.1 Nanoaperture Fabrication

The DNH is fabricated using a focused ion beam (FIB) machine. Figure 12 shows an SEM image of the specimen, a 100 nm thick gold (Au) film adhered to a 1 mm float glass test slide with a 5 nm titanium (Ti) layer (EMF Corporation). A small square (minimum size must be 9×9 mm to cover the micro-well that will be discussed in Section “3.2 Biological Solution Preparation and Sample Handling”) is cut out of the gold coated glass slide. A razor is used to mark a scratch in the middle of this sample, pointing to the region of interest (ROI) at the centre (see Figure 13). This scratch can be easily detected on the camera screen in the laser setup when shining white light through the sample (as will be discussed Section “3.3 Trapping Setup”), even if the height of the stage is not completely aligned with the focal point of the objective. The DNHs are milled as close as possible to the end of the scratch for easy detection in the trapping setup. The surface of the sample is cleaned with pure nitrogen gas to remove any residue of gold and glass (from cutting and scratching). Then the sample is ready to be placed in the FIB machine for fabrication, as can be seen in Figure 13.
Figure 12  Scanning electron microscope (SEM) image of the gold film adhered to the glass slide

Figure 13  Gold samples ready to go into the FIB machine. Two samples can be mounted on the stage holder at the same time. This allows for fabricating two different samples with the same process of aligning the machine’s beams. The fiduciary scratch is seen in the middle of the left sample, pointing to the ROI for fabrication
3.1.1 The FIB Machine

A FIB machine is a fabrication tool that is used, among other applications, for milling nanoapertures in hard materials, such as metals, semiconductors, composites, etc. Figure 14 shows a schematic of a liquid-metal ion source (LMIS) which is the most popular FIB source. In this work, a liquid gallium (Ga) ion source was used. The liquid metal is stored in a capillary tube and flows out through a tungsten needle upon heating to a melting point. The needle acts as an electrode charged with a high positive voltage. The balance between the surface tension and the electrostatic force pulls the liquid metal into a conical shape that is drawn to the small tip of the needle. The strong electric field at the tip causes field emission of gallium ions (Ga+) into a vacuum chamber. The ions are accelerated up to 40 keV while a condenser lens focuses the beam onto the surface of the substrate. By changing the energy and intensity of the beam, the surface of the specimen can be directly modified in a controlled manner\textsuperscript{78,79}.

![Figure 14 Schematic of a focused ion beam system with a liquid-metal ion source. Reprinted with permission from\textsuperscript{78} © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim](image_url)

The majority of the apertures used in this work were fabricated using the FIB machine in the Advanced Microscopy Facility at UVic- a single beam Hitachi FB-2100. Three different beams were used: 40-0-30, 40-1-80 and 40-1-15. For all three beams the
accelerating voltage is 40 keV. The middle digit in the beam name (0/1) specifies whether or not voltage is applied to the condenser lens, which leads to a high current density. The last number in the beam name specifies the size of the beam limiting aperture. The aperture size controls the amount of energy in the beam and ranges from 5 microns to 650 microns. The trade off in setting the aperture size is between the power of the beam and its resolution.

1. **40-0-30**: This is a low power beam that is used only for imaging. No voltage is applied to the condenser lens, so the current density is low. The beam limiting aperture size is 30 microns, which is relatively small. One drawback in using the gallium ion beam for imaging is that it can be distractive for the sample, especially in high magnifications. In addition, the resolution of the image is not too good due to the large size of the Ga+ (~200 pm), especially compared to the resolution of an SEM image. Using this low power beam is the only way for the user to be able to locate the ROI in the sample and to inspect the milled apertures.

2. **40-1-80**: This is a very high power beam that is used to mill the fiduciary markers in the sample. Voltage is applied to the condenser lens, so the current density is high. The beam limiting aperture size is 80 microns, which allows for more energy to come out of the ion source and enables milling through the thin gold layer and deeper into the glass slide very quickly.

3. **40-1-15**: This is the beam used to fabricate the DNHs. It is a high power beam with a relative small aperture size (15 microns) so it gives good resolution when milling.

All three beams must be carefully aligned before fabrication and this is the most time-consuming part of working with the FIB. Misalignment of the 40-0-30 beam will prevent an accurate visual inspection of the DNHs. Misalignment of the 40-1-15 beam will decrease the resolution when fabricating the apertures, and will result in a gap size that might be too wide for trapping. All three beams should also be aligned to centre at the same spot, to allow the use of the same coordinate system when moving across the specimen.
3.1.2 DNH Fabrication

The DNH is produced by milling two 180 nm circles with a 35 nm line separating them. We use a dwell time of 10 μs and 35 passes when milling. The direction of the beam (indicated as an arrow in the FIB software interface) should be set as perpendicular to the separating line axis, for better accuracy at the trapping spot. The DNH should be milled at a magnification of 20,000 times. The software interface of the FIB can be seen in Figure 15. An image of the DNH, acquired using an SEM is shown in Figure 16.

Figure 15  FB-2100 FIB machine software interface for configuring milling parameters
Before milling the final apertures at the ROI, the DNHs should be tested in a sacrificial area, and the different parameters can be slightly changed to accommodate the specific alignment of the beams. The numbers of passes (N) can vary between 25 and 40, the line length can vary between 35 and 60 nm and the circles can be moved by one or two pixels so that they will not be touching the separating line. We use the 40-0-30 beam to inspect the test DNHs at the sacrificial area and decide on the best parameters for milling. Then we move to the actual ROI and fabricate the DNHs that will be used for trapping. Even the 40-0-30 beam that is used for imaging can damage the fabricated aperture, therefore we do not inspect the DNHs at the ROI. For better accuracy, the alignment and testing should be done as close as possible to the ROI and the DNHs should be as close as possible to one another.

To make the fabricating process as efficient as possible, two gold samples can be placed in the FIB at the same time before turning it on and aligning the beams (Figure 13). The apertures have a short lifetime when used for trapping (a few weeks) so we fabricate 9 DNHs in each sample, 50 microns apart from each other (see Figure 17). This distance between the DNHs is sufficient for the laser transmission from an adjacent DNH to not
interfere while running the trapping experiment. If we expand the ROI and fabricate more than 9 DNHs, the accuracy of the aperture shape will decrease as farther we get from the centre, since the stage holder is not totally parallel to the face of the ion beam.

![SEM image](image.png)

**Figure 17**  DNHs fabricated in a gold sample. SEM image at magnification of 1715×. 9 DNHs are marked with red arrows

### 3.1.3 Fiduciary Marker Fabrication

The fiduciary marker is a big square surrounding the DNHs, making it easier to find them in the trapping setup. The 40-1-80 beam cuts deep into the glass so that the white light can pass through and the ROI can be detected on the camera screen. A fiduciary marker that is cut too close to the DNH or that is too wide or too deep, might allow for laser power to get picked up by the detector when trapping. This can affect the measurement of the transmission signal through the aperture. The square is 200×200 microns milled at a magnification of 700 times. The dwell time is 10 µs, the number of passes is 20 and the width is 0.708 microns (14 pixels). Additional smaller markers can be added outside of the square, in a set distance from the centre aperture (i.e. 200 microns as in Figure 18). These markers make it easier to find the individual DNHs when wanting to image them with the SEM.
Figure 18  SEM image of the fabricated sample at magnification of 500×. The end of the scratch (red circle) can be seen on the left and the squared fiducial marker is on the right. In the centre (blue circle) a smaller marker can be detected. This marker indicates a specific distance from the centre DNH aperture. The DNHs are too small to be seen at this magnification.

3.1.4 Challenges in Working with the Nanoapertures

Even with milling the apertures close to each other, in the same run with the same fabrication settings, a noticeable difference between the milled apertures occurs. These differences result from the large grain sizes of the gold and the FIB tolerance (6 nm) of the same order of magnitude as the DNH’s gap size. This does not allow combining trapping data acquired using different apertures, which makes it hard to gather a large amount of data for statistical analysis. The variation between two adjacent DNH apertures can be seen in Figure 19. Only after assembling the protein solution and running the experiment for a few hours, the quality of the DNH can be assessed, based on the amount of trapping events occurring.
I also had the opportunity to fabricate a few samples using a dual beam FIB (FEI Helios NanoLab 650) in 4D Labs at Simon Fraser University. That machine can accelerate the Ga+ to a maximum of only 30 keV so longer dwell time had to be used in order to cut deep enough into the glass part of the sample. Working with a dual beam machine is much less time consuming and allows for better accuracy across different apertures. A dual beam machine allows taking SEM images of the apertures without having to take the sample out and transfer it into a different machine. This is very useful when making the test DNHs. Imaging the apertures with the electron beam gives better resolution than using the low power ion beam for imaging. It also does not damage the apertures, so multiple images can be taken from different angles to determine the best parameters for fabrication. The Ga ion beam in the dual beam machine was much steadier than the one we use in the single beam machine at UVic. Even when using it for 10 hours straight, there was no need to re-align the beams. The steady beam produced samples with 9 apertures that were pretty similar to each other in shape, as can be seen in Figure 20.
Figure 20  SEM images of newly milled DNH apertures, fabricated using a dual beam FEI Helios NanoLab 650. Both apertures were milled in the same run, 150 microns apart under the same settings and have only a slight difference in gap sizes. Reprinted with permission from© 2018, American Chemical Society

After about a week of continuous trapping, the DNHs lose their shape, especially around the cusps, due to degradation of the gold surface. Trapping cannot be achieved with apertures as degraded as shown in Figure 21, and this is the main challenge in the data collection process. Multiple DNHs can be used (in sequence) in the same run of the trapping experiment for maximizing the number of events and after a few days the amount of data acquired from each aperture can be examined.

Figure 21  SEM images of a DNH aperture after continuous trapping of a week. (a) Top view. (b) 52° angle tilt. The apertures seem to lose sharpness around the cusps and edges. Reprinted with permission from© 2018, American Chemical Society
3.2 Biological Solution Preparation and Sample Handling

All protein solutions are made in a phosphate buffer solution (1xPBS) at a concentration varying between 0.01% and 1% (by weight) and stored at 4 °C. When preparing a sample for trapping, the solution is sonicated for 20 minutes to ensure uniform distribution of the protein molecules in the vile. A cover glass slide (Gold Seal, 24x60 mm, No. 0. 85-130 micron thick) is used in preparing the sample for use in the trapping setup (Figure 22a). A double-sided adhesive microscope spacer (Grace Bio-Labs, GBL654002) is used to mount the gold sample on the glass slide (Figure 22b), forming a micro-well for the solution. 10 μL of the protein solution is then micro-pipetted onto the glass (Figure 22c).

The gold sample containing the DNHs is rinsed with isopropanol, methanol, and deionized water, then dried up with nitrogen gas. The sample is adhered to the spacer, sealing the solution in the micro-well with the DNHs immersed in it (Figure 22d). The entire sample assembly is mounted in the trapping setup, on the stage between the two objectives (as shown in Figure 23).

Figure 22  Sample preparation procedure. (a) The gold sample (DNHs facing up), adhesive spacer, biological solution, no. 0 glass slide, tweezers. (b) The adhesive spacer is mounted in the centre of the glass slide. (c) The solution is pipetted into the micro-well formed by the spacer. (d) The gold sample is mounted on top of the solution. DNHs are facing down, immersed in the solution.
3.3 Trapping Setup

Figure 23  Schematic of the aperture-assisted trapping setup. Containing: a laser diode; half wave plate (HWP); polarizer; mirror (MR); beam expander (BE); dichroic mirror (DM); optical density filter (ODF); avalanche photodiode (APD); data acquisition card (DAC). Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society

Figure 23 shows the optical tweezers setup based on a Thorlabs optical tweezer kit (OTKB). The sample is held in place by a 3-axis stage between two microscope objectives. All three axes of the stage are controlled by piezoelectric elements for fine tuning.

The trapping laser is continuous with a wavelength of 852 nm (Thorlabs, DBR852P). Electromagnetic resonances of proteins are found in the frequency range of KHz through THz\textsuperscript{81}, thus the low frequency of the laser is not expected to make the trapped molecule resonate.

A half-wave plate (HWP) and a polarizer are placed in front of the fiber collimator to align the beam’s polarization to be perpendicular to the length of the DNH for maximum field enhancement, as seen in a simulation in Figure 24\textsuperscript{82}. A mirror (MR) is directing the
polarized laser into a beam expender (BE) in order to spread the beam diameter so it fills the back aperture of the objective lens.

A CCD (Charge-coupled device) camera is placed underneath the microscope objective for visualization of the DNHs in the sample and alignment with the laser beam. A dichroic mirror (DM) is placed above the camera, to reflect the laser beam into the objective. A light emitting diode (LED) is placed above the sample, transmitting white light through the DNH into the camera. This allows for visual alignment of the nanoaperture with the centre of the laser beam, as it is reflected back into the camera from the gold surface of the sample.

The laser beam is focused onto a spot that is aligned with the trapping aperture (DNH), using a 100× oil immersion condenser objective (NA=1.25). The laser power going into the microscope objective in this work was kept at 16 mW.

The light transmitted through the biological solution and the DNH is collected by a 10× objective and reflected to the left by another dichroic mirror (DM). The signal is then focused down using a lens and filtered using an optical density filter (ODF). The filtered transmission signal is detected by a silicon-based avalanche photodiode (APD) and sampled with a data acquisition card (DAC).

![Figure 24](image)

**Figure 24  Local intensity enhancement (linear scale) for a DNH of 25 nm gap and 190 nm diameter excited at 633 nm with a linear polarization parallel to the apex between the holes.** Reprinted with permission from© 2015, Springer Nature

After mounting the biosample onto the stage, the DNH should be aligned with the centre of the laser beam. The white light is used for visualization through the camera, and the first thing that is detected is the big scratch at the middle of the gold substrate. Then the X and Y axes knobs of the stage are used to find the fiduciary markers and the individual aperture.
The Z axis knob is used to bring the sample to the focal point of the condenser objective. The piezoelectric controllers of the stage can be used for fine tuning, while the indicator for the best alignment is the maximum laser transmission. When running the experiment, fine tuning of the aperture with the laser beam is needed approximately every 10 minute, due to small drifting of the stage. The drift can be noticed by a gradual decrease in the laser transmission and re-alignment is essential for a trapping event to occur.

3.4 Data Collection and Analysis

Data from the APD is sampled at a rate of 100 kHz (Advantech USB-4711A) and then downsampled by a factor of 100 by straight averaging. Figure 25a shows the signal acquired for a typical trapping event of an egg white protein. Figure 25b zooms in on the ‘untrapped’ state- the baseline transmission through the aqueous solution, where there is no particle in the trap (the DNH). We can see that downsampling is reducing the detection noise significantly.

Figure 25  Trapping event of an egg white protein. Blue curve shows the raw data, sampled at 100 kHz. Red curve shows the data downsampled to a frequency of 1 kHz. (a) The untrapped state and the trapped one are indicated. Trapping is identified from a sudden increase in transmission. (b) Zoom in on the untrapped signal. (c) Zoom in on the trapped signal
When a molecule gets trapped, we notice a distinct increase in the laser transmission, due to the dielectric loading of the nanoaperture with the molecule within it. The next segment of the data is referred to as the ‘trapped’ state, and we can notice a significant increase in the signal fluctuations, due to the molecule’s movement in the trap. Figure 25c zooms in on the trapped state, and we can see the large amount of fluctuations even when averaging out the system’s noise.

A globular protein can be approximated as a sphere\(^\text{83}\), with a polarizability (\(\alpha\)) that scales as its radius to the third power (Equation 5). Even though the proteins are not perfectly spherical, similar formulations are used for dielectrophoresis and have been shown to work in practice\(^\text{84}\). The trapping potential (\(U\)) is proportional to the volume of the molecule (\(r^3\)), as stated in Equation (17). The intensity of the transmission (\(I\)) picked up by the detector is proportional to the potential energy, so the signal intensity is expected to scale with the molecule’s volume. Assuming the same mass density for all proteins in our comparison, the volume will scale with the molecular mass, giving:

\[
I \propto V \propto M_r
\]  

(21)

The fluctuations in the signal intensity result from the Brownian motion of the particle in the trap. This motion under the influence of thermal fluctuation and viscose drag force can be modeled by an overdamped Langevin equation as\(^\text{85}\):

\[
\frac{dx(t)}{dt} = \frac{\kappa}{\gamma} x(t) + \sqrt{\frac{2k_BT}{\gamma}} \xi(t)
\]  

(22)

Where \(x(t)\) is the particle’s displacement from the equilibrium position, \(\kappa\) is the trap stiffness, \(\gamma\) is the Stokes’ drag coefficient, and \(\xi(t)\) is white noise.

Using Equation (22) we can define a time constant (\(\tau\)), that is used in conventional optical traps as a measure of the trapping stiffness\(^\text{85–87}\):

\[
\tau = \frac{\gamma}{\kappa}
\]  

(23)

The trap stiffness (\(\kappa\)) relates the gradient force to the displacement (\(x\)), just like a spring constant, as discussed in Section “2.1 Rayleigh Scattering and Conventional Optical Tweezers”.
\[ \tau = 6\pi\eta r \cdot \frac{dx}{-dF_{\text{grad}}} \]  

(24)

Where \( \eta \) is the viscosity of the medium. The gradient force scales as the third power of the radius (Equation 15), and the exponential time constant scales with \( \frac{1}{r^2} \):

\[ \tau \propto \frac{r}{r^3} \propto V^{-\frac{2}{3}} \propto M_r^{-\frac{2}{3}} \]  

(25)

We find the time constant by computing the autocorrelation function (ACF) of the trapped signal, and examining its exponential decay time\(^{86}\). The autocorrelation of a signal measures the correlation between values of it at different times, as a function of the time-lag between these times:

\[ R(lag) = \frac{\sum_{t=1}^{N-lag}(f(t)-\bar{f})(f(t+lag)-\bar{f})}{N\sigma^2} \]  

(26)

Where \( N \) is the length of the signal and \( \sigma^2 \) is its variance.

Figure 26 shows the ACFs of both the untrapped and trapped states. The blue curve shows an immediate drop in correlation coefficients for the untrapped state while the red one displays an exponential decay as lag time extends.

In this work the ACF of trapped signals was found to fit well with a two-exponent function (dashed black line in Figure 26). The stiffness of the trap, arising from the laser gradient force, can be estimated by the time constant resulting from the Brownian motion of the protein within the trap (Equation 23). For that purpose we use the faster time scale of the exponential decay.

The slower time constant that was found here for the first time, can be explained by the conformational changes of the proteins, as was observed in previous work\(^{32,83}\). This time constant was not considered for the analysis in this thesis.
Figure 26  *Autocorrelation function for a trapping event of an egg white protein.* ACF of the untrapped (blue) and trapped (red) states with a two-exponent function fit to ACF of the trapped signal (dashed black). Reprinted with permission from© 2018, American Chemical Society

The DNH tweezers were used in a previous work for characterizing the molecular weight of single globular proteins, using information harvested from the trapped signal. This method was also used in this thesis for detecting specific proteins out of heterogeneous mixtures. Based on the relation in (21) we expect the standard deviation (SD) of the trapped signal to have a linear dependence on the molecular mass. Using the relation in (25) we expect the faster time constant (τ) of the autocorrelation function (ACF) to have a -2/3 power dependence on molecular mass. Furthermore, we plot the SD in respect to τ for the different trapping events, and expect a -2/3 power dependence.

The SD in this work was calculated for a 5 second segment out of the trapped signal and was divided by the mean value of the untrapped signal for normalization. The ACF was calculated for a 1 minute segment of trapped data and was then fit to a two-exponent function using a non-linear least squares fit. The fast time constant (τ) was recorded for each trapping event.
In most cases the molecule will stay trapped until we block the laser for a short period of time. The optical power is able to overcome the Brownian motion for a long time (up to 30 minutes were tried). Usually when the particle stays trapped for more than a couple of minutes, the laser has to be blocked for a significant amount of time (~10 minutes) in order to get it out of the trap. This most likely happens due to sticking of the protein to the edges of the gold surface, and can be seen in Figure 27a. Sometimes the molecule gets physically stuck and the sample must be taken apart and cleaned thoroughly in order to run the experiment again. This happens mostly with larger molecules.

Figure 27b shows a second jump (increase in transmission) within the trapped state. We attribute that to a second molecule that enters the trap. These events were not studied in this work and have been excluded from the data collected. In cases where the second trapping event happened more than 30 seconds after the first one, the first trapping event was analyzed as usual and the data after the second jump was disregarded.

![Figure 27](image)

**Figure 27** Examples of disregarded data, while trapping egg white proteins. (a) The signal looks noisy as soon as the laser power is back on, probably due to a molecule stack close to the trap. The signal increases gradually, then stabilizes and looks like a normal trapping event. (b) A second jump in the laser transmission after the first trapping event. We assume this is a result of a second molecule entering the trap. Reprinted with permission from© 2018, American Chemical Society
In rare cases we observe a trapping event that lasts only a few seconds, where the signal spontaneously drops back down to the untrapped voltage level. These events were ignored in this work.

3.5 Summary

The experimental methods for aperture assisted optical trapping were presented in this chapter. The most challenging step is the aperture fabrication one, as explained in Section “3.1.4 Challenges in Working with the Nanoapertures”. Working with the FIB machine has many variabilities and there are uncertainties regarding the quality of the trapping signal that can be achieved when using the aperture. Correct handling of the biosample should be used to prevent contamination and denaturing of the proteins. The trapping laser setup should always be aligned properly for successful trapping.
Chapter 4 - Results and Discussion

This work focuses on the DNH optical tweezers’ ability to distinguish proteins in a heterogeneous mixture by examining the characteristics of the laser signal during a trapping event of a single molecule. As a proof of concept, egg white was chosen to be the mixture for protein composition analysis. The protein content of egg white has been studied greatly using different techniques and is well known. This allows validating the results of the content analysis using the aperture tweezers, via a comparison with the known content of egg white protein.

As discussed in Section “2.3 DNH Optical Tweezers Application for Single Biomolecule Studies”, our technique has been proven in the past as a successful tool for studying protein single molecule characteristics and investigating protein-protein interactions, protein-small molecule interactions, and protein-DNA interactions. These experiments were done using aqueous biosamples prepared with clean and purified protein powders, professionally made for laboratory work, where only one or two studied proteins were present. The goal in this current work was to explore the trapping signals of biomolecules in ‘dirty’ conditions, when trapped out of a solution containing different proteins, as well as other ingredients like sugars and fats.

First, two size-standard proteins that are known to be found in egg white (ovotransferrin and ovalbumin) were trapped individually. The results are reported in Section “4.1 Trapping of Size Standard Proteins”. This reproduces previous results that were published by our group\(^8\). It also helps validating the method for evaluating the molecular weight of the protein, based on the trapping signal characteristics, as presented in Section “3.4 Data Collection and Analysis”.

Next, as a controlled heterogeneous mixture, the same trapping analysis was done for a solution containing the same two proteins (ovotransferrin and ovalbumin) in a 1:1 ratio. Trapping events from this mixed solution show similar results, as described in Section “4.2 Trapping of a Mixed Solution”. Finally, an egg white solution was made using a store-bought egg. The experimental results of trapping molecules out of the egg white solution are presented in Section “4.3 Egg White Analysis”. Grouping of these trapping events in
the SD vs. \( \tau \) plot, suggests similarity between the composition analysis of this heterogeneous mixture and the known protein composition of egg white.

### 4.1 Trapping of Size Standard Proteins

The first step in this work was to validate the theory presented in Section “3.4 Data Collection and Analysis” by analyzing the trapping events of two size standard proteins: ovotransferrin (\( M_r = 77.7 \text{ kDa} \)) and ovalbumin (\( M_r = 44.5 \text{ kDa} \)). This type of analysis was done in the past with 6 different size standard proteins\(^8\). The results of this previous study (see Figure 28) show a positive linear relationship between the molecular mass of the protein and the fluctuations of its trapped signal. They also show a modeled \(-2/3\) power dependence between the molecular mass of the protein and the time constant of its trapped signal ACF.

![Figure 28](image)

**Figure 28**  RMS variation of trapped particles, autocorrelation of trapped particles. (a) RMS of the trapped particles with respect to their molecular weight. (b) Autocorrelation relaxation time for the trapped particles with respect to their molecular weight. Reprinted with permission from\(^8\) © 2015, the Royal Society of Chemistry

Figure 28b shows that the time constant calculated for ovotransferrin (\( M_r = 77.7 \text{ kDa} \)) did not follow the predicted model. We note that in that publication, the ACF was fit to a one-
exponent curve (instead of a two-exponent curve that was found more accurate in this current work).

Ovotransferrin acts as the iron transporter in the egg. It folds into two loosely connected globular lobes, each containing an iron-binding site. The large time scale fluctuations may be the result of those conformational changes of the molecule, while it is trapped. A supporting evidence for this hypothesis can be detected in the distribution of the trapping data obtained for ovotransferrin in the same publication. Rather than a single Gaussian peak, a bimodal distribution can be seen, as shown in Figure 29. As explained in Section “3.4 Data Collection and Analysis”, when analyzing the relaxation time of the ACF for the purpose of characterizing the molecular weight, the time constant of interest is the faster one. That $\tau$ result from the movement of the molecule in the trap and is related to its stiffness.

![Figure 29](image.png)

**Figure 29** Histograms of trapping data. Bimodal distribution of voltage values for ovotransferrin. Reprinted with permission from © 2015, the Royal Society of Chemistry

These two size standard proteins (ovotransferrin and ovalbumin) were chosen for the first step of this work since they are known to be found in egg white. Figure 30a shows the trapping data of both proteins in an SD vs. $\tau$ plot. Clustering of the data can be noticed for each one of the proteins. From the dependence of the signal’s intensity on the molecular mass (21), we expect ovotransferrin ($M_r = 77.7$ kDa) to have higher SD values than
ovalbumin (M_r = 44.5 kDa). From the dependence of the time constant on the molecular mass (25), we expect ovotransferrin to also have lower τ values than ovalbumin.

Figure 30b shows an error bar plot of these events, as a mean ± 1 standard deviation of the SD’s of the trapping events for the proteins. The two points were fitted to a \( y = 0.05x^{-0.74} \) curve, which shows better agreement with the -2/3 dependence that was predicted by the theoretical model.

The first step of this work was able to verify the previous results relating the SD and τ values of the trapping data to the molecular mass of the protein. The change in the exponential curve fit (a two exponent function instead of a one exponent function) proved to be more accurate compared with a previous study.

### 4.2 Trapping of a Mixed Solution

For the second step of this work, a solution was made with a mixture of 50% ovalbumin and 50% ovotransferrin (by volume). Figure 31a shows the SD as a function of τ for all of the individual trapping events in this artificial mixture. These results can be separated into
two groups. The black triangles (group A) are classified as ovotransferrin trapping events and the blue circles (group B) are classified as ovalbumin events. The ranges of SD and \( \tau \) values in the groups seems to correlate with the ranges found for the pure proteins trapping events (Figure 30). The equal number of trapping events in each group also fit with the 1:1 ration of the two proteins in the solution. Figure 31b shows an error bar plot of these events, as a mean ± 1 standard deviation of the SD’s of the trapping events for the proteins. The two points were fitted to a \( y = 0.05x^{-0.81} \) curve.

![Figure 31](image)

**Figure 31** Standard deviation (SD) and autocorrelation function time constant (\( \tau \)) of ovotransferrin and ovalbumin trapping events. (a) A mixed solution of ovotransferrin and ovalbumin (1:1 ratio by volume). Group A was classified as ovotransferrin particles; group B was classified as ovalbumin particles. (b) Mean ± 1 standard deviation of group A (black) and group B (blue) SD results. The two data points were fitted to \( y = 0.05x^{-0.81} \) (red). Reprinted with permission from 35 © 2018, American Chemical Society

### 4.3 Egg White Analysis

The goal of this experimental work was to implement the trapping signals’ characterization for analyzing the egg white protein content. We used a store-bought egg to prepare the solution for trapping, and verified the results by a comparison with the known protein content of egg white. Chicken egg white is composed of: 87.9% water; 10.6% proteins; 0.03% fat. 0.9% carbohydrates; and 0.6% minerals. More than 70 different proteins can
be identified in egg white\textsuperscript{90}, the main 13 of them are presented in Table 1, along with their molecular mass and abundance in chicken egg white, as found in the literature.

**Table 1: Main proteins known to be found in egg white\textsuperscript{89,91}**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>M\textsubscript{r} (kDa)</th>
<th>Abundance in egg white (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ovomucin</td>
<td>5.5-8.3\texttimes10\textsuperscript{6}</td>
<td>3.5</td>
</tr>
<tr>
<td>ovomacroglobulin</td>
<td>760-900</td>
<td>0.5</td>
</tr>
<tr>
<td>ovotransferrin</td>
<td>77.7</td>
<td>12</td>
</tr>
<tr>
<td>avidin</td>
<td>68.3</td>
<td>0.05</td>
</tr>
<tr>
<td>ovoinhibitor</td>
<td>49.0</td>
<td>1.5</td>
</tr>
<tr>
<td>ovoglobulin G3</td>
<td>49.0</td>
<td>4</td>
</tr>
<tr>
<td>ovalbumin</td>
<td>44.5</td>
<td>54</td>
</tr>
<tr>
<td>ovoglobulin G2</td>
<td>36.0</td>
<td>4</td>
</tr>
<tr>
<td>flavoprotein</td>
<td>32.0</td>
<td>0.8</td>
</tr>
<tr>
<td>ovomucoid</td>
<td>28.0</td>
<td>11</td>
</tr>
<tr>
<td>ovoglycoprotein</td>
<td>24.0</td>
<td>1</td>
</tr>
<tr>
<td>lysozyme</td>
<td>14.3</td>
<td>3.4</td>
</tr>
<tr>
<td>cystatin</td>
<td>12.7</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Proteins found in concentrations of 1.5% or less were neglected in this work, due to the low chances of trapping them. Ovomucin was neglected as well, due its size, which is too big to enter the DNH trap. The protein’s molecules are approximated as spheres with an estimated specific volume of \textsuperscript{92}:

\[
V = 0.73 \frac{cm^3}{g} = 1.21 \frac{nm^3}{kDa}
\]  

(27)

Based on Equation (27) and assuming a gap size of 20nm (Figure 33), the maximum molecular weight of a particle that can be trapped is M\textsubscript{r} \approx 3400kDa.

After neglecting these from our analysis, we are left with 6 main proteins of egg white. Those proteins were divided into 3 groups based on their molecular weights, as described on the right in Table 2.
Figure 32a presents the SD vs. $\tau$ plot for 25 egg white trapping events. All events were obtained with the DNH shown in Figure 33. Three groups can be detected in the distribution of the data. We attribute the black triangles (Group A) to proteins with a molecular mass greater than 49 kDa, the blue circles (Group B) to proteins with a molecular mass ranging between 36-49 kDa and the green squares (Group C) to proteins with a molecular mass smaller than 36 kDa. In Figure 32b the same SD and $\tau$ values are plotted on a log-log scale. A linear curve ($y = -0.634x - 1.59$) was fit to the results in this graph (dashed red). The slope of the fitted curve shows good agreement with the scaling of mass to the -2/3 power to $\tau$ and linearity to SD, with an error of only 4.8%.

Figure 32  Standard deviation (SD) and autocorrelation function time constant ($\tau$) of egg white trapping events. Trapping events in group A (black) were classified as particles with a molecular weight higher than 49 kDa; trapping events in group B (blue) were classified as trapped particles with a molecular weight in the range 36–49 kDa; trapping events in group C (green) were classified as trapped particles with a molecular weight lower than 39 kDa. (a) SD vs. $\tau$ plot. (b) log(SD) vs. log($\tau$) plot. The distribution is fit to $y = -0.634x - 1.59$ (red). Reprinted with permission from$^{35}$ © 2018, American Chemical Society
Table 2 shows the percent abundances of trapped and classified egg white proteins (as seen in Figure 32), compared to the grouping of the 6 main proteins in egg white from the literature. Errors between the percent abundance of each group is noted, but the number of events is also kept in mind as a factor reflecting this variance. The agreement of the egg white proteins trapped with the abundance in typical chicken egg white is evident.

**Table 2: Classification of egg white protein composition.** Left: Grouping of experimental data (as shown in Figure 32), and classification by molecular weight ($M_r$). Right: Main proteins found in egg white, grouping by molecular weight, and scaled abundance in egg white. Reprinted with permission from © 2018, American Chemical Society

<table>
<thead>
<tr>
<th>Group</th>
<th>$M_r$ range (kDa)</th>
<th>Number of events</th>
<th>Percentage</th>
<th>Protein Name</th>
<th>$M_r$ (kDa)</th>
<th>Abundance in egg white</th>
<th>Scaled abundance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$49 &lt; M_r$</td>
<td>2</td>
<td>8%</td>
<td>ovotransferrin</td>
<td>77.7</td>
<td>12%</td>
<td>12%</td>
</tr>
<tr>
<td>B</td>
<td>$36 &lt; M_r &lt; 49$</td>
<td>19</td>
<td>76%</td>
<td>ovoglobulin G3</td>
<td>49.0</td>
<td>4%</td>
<td>62%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ovalbumin</td>
<td>44.5</td>
<td>54%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ovoglobulin G2</td>
<td>36.0</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>$M_r &lt; 36$</td>
<td>4</td>
<td>16%</td>
<td>ovomucoid</td>
<td>28.0</td>
<td>11%</td>
<td>14.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lysozyme</td>
<td>14.3</td>
<td>3.4%</td>
<td></td>
</tr>
</tbody>
</table>

*neglecting proteins with low concentrations (Abundance ≤1.5%) and high mass ($M_r > 3400$ kDa)
Time series data and autocorrelation functions of individual trapping events signals can be seen in Figure 34 through Figure 39. Two typical trapping events are presented out of each egg white groups (A, B & C; Figure 32), as well as out of the pure proteins ovotransferrin and ovalbumin. For comparison with the lower mass group of egg white proteins (Group C), we also ran the experiment with a pure ovomucoid protein solution. Ovomucoid has a molecular mass of $M_r = 28 \text{kDa}$ and is known to compose of 11% out of the protein content in egg white. Two typical trapping events of ovomucoid can be seen in Figure 38. These trapping events were acquired using a different DNH, therefore the ovomucoid SD and $\tau$ values could not be compared with those of the pure proteins or the egg white.

Visual inspection of the egg white proteins’ trapping events and those of the pure proteins shows matching characteristics. SD amplitudes and time constant analysis give similar values among the same protein. Variations in trapped data for the same group of egg white proteins are seen, but those variations are also present in signals of the pure proteins, and we attribute them to natural variations among the protein molecules.
Figure 34  Trapping events of pure ovotransferrin molecules. (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.19e^{-t/4.69} + 0.01e^{-t/1380}$ (red).

(b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.08e^{-t/5.21} + 0.08e^{-t/195}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from$^{35}$ © 2018, American Chemical Society
Figure 35  Trapping events of pure egg white protein molecules from Group A (Figure 32). (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.35e^{-t/2.8} + 0.03e^{-t/153}$ (red). (b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 1.19e^{-t/3.91} + 0.06e^{-t/164}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from 35 © 2018, American Chemical Society.
Figure 36  Trapping events of pure ovalbumin molecules. (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.7e^{-t/23.4} + 0.3e^{-t/183}$ (red).
(b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.9e^{-t/6.85} + 0.17e^{-t/103}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society
Figure 37  Trapping events of pure egg white protein molecules from Group B (Figure 32).

(a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.77e^{-t/13.8} + 0.22e^{-t/383}$ (red).

(b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.79e^{-t/987} + 0.29e^{-t/71.3}$ (red).

Time constants ($\tau$) are given in milliseconds. Reprinted with permission from $^{35}$ © 2018, American Chemical Society
Figure 38  Trapping events of pure ovomucoid molecules. (a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.7e^{-t/23.36} + 0.3e^{-t/186}$ (red).

(b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.9e^{-t/16.3} + 0.17e^{-t/632}$ (red). Time constants ($\tau$) are given in milliseconds. Reprinted with permission from\textsuperscript{35} © 2018, American Chemical Society
Figure 39  Trapping events of pure egg white protein molecules from Group C (Figure 32).
(a) Left: normalized time series of a trapped signal. (a) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.58e^{-t/27} + 0.33e^{-t/320}$ (red).
(b) Left: normalized time series of another trapped signal. (b) Right: autocorrelation function of the trapped signal (blue). ACF is fitted with the function $y = 0.58e^{-t/21.75} + 0.37e^{-t/439}$ (red).
Time constants ($\tau$) are given in milliseconds. Reprinted with permission from$^{35}$ © 2018, American Chemical Society
4.4 Summary

The first two parts of the experiment were done using the same DNH, therefore the results of the separated ovotransferrin and ovalbumin trapping events are in agreement with the trapping results of the mixed solution. More trapping events out of the mixed solution would have been desirable but no more trapping events could be achieved at that point. When running the egg white trapping experiment, the goal was to get as many trapping events as possible, therefore no pure proteins were trapped using the same DNH. Ovomucoid was trapped as well in this work, as another reference to the trapped signals acquired from egg white.

These results demonstrate the ability of aperture tweezers to isolate single proteins in egg white, classify them based on mass, and to determine relative concentrations of the proteins in egg white. This is the first time that this technique is used for the analysis of a dirty sample. Ideally, a much larger amount of data should have been collected, but the lifetime of the nanoapertures used in this work was a significant limiting factor (as discussed in Section “3.1.4 Challenges in Working with the Nanoapertures”). This is the first step in enabling the use of the tweezers for studying single molecules in heterogeneous protein mixtures.
Chapter 5 - Summary and Future Work

This thesis summarizes the work done with the DNH optical tweezers for studying single proteins. It also describes the personal challenges and setbacks encountered during this work, and provides a ‘recipe’ for the procedure that was personally found successful for running the aperture assisted optical trapping experiment.

In addition to reproducing past results and slightly revising the data analysis method, the work presented in this thesis studied the trapping signals of egg white proteins. This work is the first one to use the DNH tweezers for exploring heterogeneous solutions. It was shown that this technique can be used for analyzing the protein composition of an unprocessed solution, in good agreement with the known composition from the literature.

5.1 Conclusions

This technique enables real-time studies of proteins at the molecular level, in a label-free free-solution environment. Making it a useful tool for the biomedical research community and an effective one for drug development, will require its ability to be used with heterogeneous mixtures. DNH optical trapping is currently used for probing the dynamic behaviour of specific proteins and their various ways of interacting with one another. The ability to detect and distinguish a protein once trapping it out of a ‘dirty’ sample would allow for major advancements in the tweezers’ applications. The analysis method presented here will probably need to be improved in its ability to distinguish between proteins with similar molecular mass, and the throughput will have to increase significantly.

The results of this work are the first step towards studying unprocessed physiological solutions like blood, saliva and other body fluids using the DNH optical tweezers. Detecting biomarkers with this technique for example, could contribute to personalized and preventative medicine, as well as to diagnosis of pathologies and monitoring of disease progression.
5.2 Future Work

One of the challenges when working with this experimental setup is the difficulty in collecting a large amount of data using the same nanohole. Therefore any quantitative study cannot involve a big sample size. Moreover, it was found that different apertures have different efficiencies in trapping different proteins. Developing a data normalization method that will allow to compare results acquired from different DNHs would have a big impact on the amount of data points that could be analyzed in a single work. It would also allow for using all 9 apertures fabricated in the same sample, by switching back and forth when a particle gets stuck in one of the DNHs. This will increase the potential number of trapping events acquired, and will delay the degradation of the apertures.

Another important factor that affects the nature of the data collection process, is the complexity and sensitivity of the laser setup. The trapping setup includes many optical components that need to be carefully aligned to maximize the optical power reaching the sample. In addition, any small drift of the sample holder prevents trapping from occurring, so the piezoelectric elements of the stage must be repeatedly manually adjusted. A solution for these issues could come from integrating the DNH aperture with an optical fiber. This would simplify the laser system, making it portable, easier to use, and significantly cheaper. It would also enable the simultaneous use of multiple fibers in the same biological solution, increasing the throughput of the system. This approach would also allow for transportation of the trapped particles after characterizing the specific one that got trapped.

Recent work that was published by our group\textsuperscript{93} has demonstrated the ability to trap 20 nm polystyrene particles using a nanoaperture fiber tweezer (NAFT). A template-stripping method was used to integrate the nanoaperture at the tip of the optical fiber. This approach makes the fabrication process more robust, since it is not done on the fiber itself, rather on a separate gold sample that is later epoxied onto the tip of the fiber. Incorporating this technique with the protein work presented in this thesis has the potential of making the tweezers applicable and far more accessible for many research laboratories and industrial users as well.
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doi:10.1103/PhysRevLett.95.168102


Appendix - Matlab Code

```matlab
function trapAnalysis(path, fs)
    ds = 100;
    if path(end) ~= '\'
        path = [path ' \'];
    end
    txtFiles = dir(fullfile(path, '*.txt'));
    fileNames = extractField(txtFiles, 'name');
    std4path = [];
    tau4path = [];
    for i = 1:length(fileNames)
        fileName = fileNames{i}; fileName = fileName(1:end-4);
        fileName(fileName == '_') = '-';
        % read data from text file:
        A = importdata(fullfile(path, fileNames{i}), ' ', 15);
        data = A.data;
        figure; plot(1/fs:1/fs:length(data)/fs, data);
        bInd = str2double(cell2mat(inputdlg('Choose the BEGINNING time index')));
        eInd = str2double(cell2mat(inputdlg('Choose the END time index. Enter 0 to ignore this text file')));
        close
        if eInd
            data = data(bInd*fs+1:eInd*fs);
            winData = reshape(data(1:floor(length(data)/ds)*ds), [ds, floor(length(data)/ds)]);
            avgData = mean(winData);
            % find the jump and plot the data:
            diffData = diff(avgData);
            [xx, maxInd] = max(diffData); jumpInd = round(maxInd/(fs/ds));
            h = figure; subplot(2, 1, 1); hold on
            suptitle(fileName)
            if the jumped occurred less than 5 sec after the beginning of the recording:
                if jumpInd <= 5
                    plot(1/(fs/ds):1/(fs/ds):10, avgData(1:20*(fs/ds)));
                    axis tight
                    utInd = str2double(cell2mat(inputdlg('Insert time index for UNTRAPPED state beginning')));
                    untrapped = avgData(utInd*(fs/ds)+1:(jumpInd-1)*(fs/ds));
                else
                    plot((jumpInd-10):1/(fs/ds):min((jumpInd+10), length(data)/fs), data((jumpInd-10)*fs:min((jumpInd+10)*fs, length(data)));
                    plot((jumpInd-10):1/(fs/ds):min((jumpInd+10), length(avgData)/fs), avgData((jumpInd-10)*fs:min((jumpInd+10)*fs, length(avgData)));
                    axis tight
                    % get time indices for untrapped and trapped sections:
                    utInd = str2double(cell2mat(inputdlg('Insert time index for UNTRAPPED state beginning')));
                    untrapped = avgData(utInd*(fs/ds)+1:(utInd+5)*(fs/ds));
                end
            end
            tInd = str2double(cell2mat(inputdlg('Insert time index for TRAPPED state beginning')));
            xlabel('Time [s]'); ylabel('Laser Transmission [V]');
            legend('raw', 'downsampled', 'Location', 'NW')
            trapped = avgData(tInd*(fs/ds):end);
        end
```

% standard deviation:
std_data = std(trapped(1:5*(fs/ds))/mean(untrapped));
title(['STD=' num2str(std_data)])

% autocorrelation
acf_trapped = acf(trapped(1:min(60*(fs/ds), length(trapped))), 1001);
acf_fit = fit((1/(fs/ds):1/(fs/ds):length(acf_trapped)/(fs/ds))',
               acf_trapped, 'exp2');
t1 = 1000/-(acf_fit.b); t2 = 1000/-(acf_fit.d);
tau = min(t1, t2);

% plot ACF:
subplot(2,1,2); hold on
plot(1/(fs/ds):1/(fs/ds):length(acf_trapped)/(fs/ds), acf_trapped)
plot(acf_fit, 'k--')
axis([0 length(acf_trapped)/(fs/ds) 0 1]);
xlabel('Time [s]'); ylabel('Autocorrelation')
title(['TAU=' num2str(tau) ' msec'])

std4path = [std4path; std_data];
tau4path = [tau4path; tau];
save([path fileName '.mat'], 'fs', 'data', 'avgData', 'utInd',
     'tInd', 'untrapped', 'trapped', std_data', 'acf_trapped', 'tau');
saveas(h, [path fileName '.jpg']);
close clear data avgData
end

save([path 'data'], 'std4path', 'tau4path');
% plot STD-TAU:
h = figure;
plot(tau4path, std4path, '*','markersize', 10)
xlabel('ACF tau [ms]'); ylabel('Normalized STD')
saveas(h, [path 'data.jpg']);
function ta = acf(y,p)
[n1, n2] = size(y) ;
if n2 ~=1
    error('Input series y must be an nx1 column vector')
end
[a1, a2] = size(p) ;
if ~(a1==1 & a2==1) & (p<n1)
    error('Input number of lags p must be a 1x1 scalar, and must be
          less than length of series y')
end
ta = zeros(p,1) ;
global N
N = max(size(y)) ;
global ybar
ybar = mean(y);

% Collect ACFs at each lag i:
for i = 1:p
    ta(i) = acf_k(y,i-1) ;
end
% ----------------------
function ta2 = acf_k(y,k)
global ybar
global N
cross_sum = zeros(N-k,1) ;
% Numerator, unscaled covariance:
for i = (k+1):N
    cross_sum(i) = (y(i)-ybar)*(y(i-k)-ybar) ;
end
% Denominator, unscaled variance:
yvar = (y-ybar)'*(y-ybar) ;
ta2 = sum(cross_sum) / yvar ;