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Establishment of a multiple particle tracking video microscopy protocol for characterization of nanoparticle transport in complex biological environments

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Abstract

Nanotechnology has been the focus of considerable study for several decades. Its improvements keep attracting the researchers' attention. There are many applications to different nanosystems. In the drug delivery systems, nanoparticles have been studied for decades with substantial advances. Different techniques have been devised to study the properties of nanoparticles, namely their interactions with biological structures. Understanding the behavior and transport of nanoparticles in complex biological environments is important for the purpose of medical application, and multiple particle tracking (MPT) video microscopy can be of help for these last objective. These studies comprise several and diverse phases, such as nanoparticle solution preparation, video acquisition, video analysis (to extract nanoparticle motion data) and data analysis. The last phase, data analysis, has the great impact in terms of research time and effort, in which the nanoparticle trajectories are analyzed and characterized. Therefore, it is important to define a protocol to establish a study pattern and improve the research outputs.

The study of nanoparticle motion in complex biological media using MPT analysis provides valuable hints for understanding the physics of particle transport and the structural features of the surrounding environment. Transport can be characterized in media such as biological fluids, tissues, or even single cells. Trajectories are extracted using algorithms commonly available in image processing software (e.g., the particle-tracking plugin of ImageJ) by linking the centroid of nanoparticles between subsequent frames of short-duration videos. However, subsequent manual conversion of trajectories into relevant transport parameters is a tedious process that involves iterative calculations. The procedure is time consuming and prone to user-related errors and requires substantial computing resources. There are several applications and program scripts to help performing MPT data analysis. Nonetheless, not all of them are sufficiently fast and user-friendly.

Thus, we developed a new, fast, and user-friendly software comprising a Graphical User Interface on top of a free Python library (TrackPy). The software allows analyzing tracking data and calculating mean squared displacement (MSD) and effective diffusivity values of sub-micrometer particles based on their two-dimensional coordinates. This tool is further able to extract values of time-independent diffusion coefficient and anomalous diffusion exponent from MSD vs. time-scale plots, and to categorize individual and ensemble particle transport as immobile, sub-diffusive or diffusive. In order to demonstrate the usefulness of the software, we tested and validated it by analyzing trajectories collected from video microscopy of carboxyl-functionalized polystyrene particles (100-200 nm) dispersed in artificial intestinal mucus. The software was shown able to accurately process large datasets and provide numerical/graphical representation of different variables within a few seconds. Indeed, the proposed protocol, using the newly developed application, the overall data analysis time decreased from over 5 hours to less than one minute, allowing faster test iterations. Overall, the proposed software has the potential to be used in high-throughput analysis of nanoparticle transport in biorelevant media.

Resumo

A nanotecnologia tem sido o foco de muitos estudos por várias décadas. As suas melhorias continuam a atrair a atenção dos investigadores. Há muitas aplicações para diferentes nanosistemas. Nos sistemas de administração de medicamentos, as nanopartículas têm sido estudadas há décadas com avanços substanciais. Foram concebidas diferentes técnicas para estudar as propriedades das nanopartículas, nomeadamente as suas interações com estruturas biológicas. A compreensão do comportamento e transporte de nanopartículas em ambientes biológicos complexos é importante para efeitos de aplicação médica, e o rastreio de múltiplas partículas (MPT) por microscopia de vídeo pode ser útil para estes últimos objectivos. Estes estudos compreendem várias e diversas fases, tais como preparação de soluções de nanopartículas, aquisição de vídeo, análise de vídeo (para extrair dados de movimento de nanopartículas) e análise de dados. A última fase, a análise de dados, tem grande impacto em termos de tempo e esforço de investigação, na qual as trajectórias das nanopartículas são analisadas e caracterizadas. Por conseguinte, é importante definir um protocolo para estabelecer um padrão de estudo e melhorar os resultados da investigação.

O estudo do movimento de nanopartículas em meios biológicos complexos utilizando a análise MPT fornece dicas valiosas para a compreensão da física do transporte de partículas e das características estruturais do ambiente circundante. O transporte pode ser caracterizado em meios como fluidos biológicos, tecidos, ou mesmo células únicas. As trajectórias são extraídas usando algoritmos normalmente disponíveis em programas de processamento de imagem (por exemplo, o plugin de rastreio de partículas do ImageJ), ligando o centróide das nanopartículas entre quadros subsequentes de vídeos de curta duração. No entanto, a subsequente conversão manual de trajectórias em parâmetros de transporte relevantes é um processo tedioso que envolve cálculos iterativos. O procedimento é moroso e propenso a erros relacionados com o utilizador e requer recursos informáticos substanciais. Há várias aplicações e scripts de programas para ajudar a realizar a análise de dados de MPT. No entanto, nem todas elas são suficientemente rápidas e fáceis de utilizar.

Assim, desenvolvemos um software novo, rápido, e de fácil utilização, compreendendo uma Interface Gráfica sobre uma biblioteca Python livre (TrackPy). O software permite analisar os dados de rastreio e calcular o deslocamento médio quadrático (MSD) e os valores efectivos de difusividade das partículas sub-micrómicas com base nas suas coordenadas bidimensionais. Esta ferramenta é ainda capaz de extrair valores de coeficiente de difusão independente do tempo e expoente de difusão anómala do MSD versus parcelas de escala temporal, e de categorizar o transporte individual e de conjunto de partículas como imóvel, sub-difusivo ou difusivo. A fim de demonstrar a utilidade do software, testámos e validámos-lo analisando trajectórias recolhidas a partir de microscopia vídeo de partículas de poliestireno carboxil-funcionalizado (100-200 nm) dispersas em muco intestinal artificial. O software revelou-se capaz de processar com precisão grandes conjuntos de dados e fornecer representação numérica/gráfica de diferentes variáveis em poucos segundos. De facto, no protocolo proposto, utilizando a aplicação recentemente desenvolvida, o tempo global de análise de dados diminuiu de mais de 5 horas para menos de um minuto, permitindo iterações de teste mais rápidas. Globalmente, o software proposto tem o potencial de ser utilizado na análise de alto rendimento do transporte de nanopartículas em meios bio-relevantes.

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Just as nanoparticles move in complex biological environments, so we move in a complex world with many challenges. Overcoming them is not always easy, but we find people along the way who help us in this task. The challenge that I set myself, to resume my studies after almost two decades, was only overcome with the help of many people, who contributed in the most diverse ways.

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Leandro de Assis Gabriel

*“Anyone who stops learning is old, whether at twenty or eighty.
Anyone who keeps learning stays young.
The greatest thing in life is to keep your mind young.”*

Henry Ford

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Abbreviations and Symbols

List of abbreviations

3D	Three dimensional
AI	Artificial Intelligence
COOH-PS	Carboxylate-modified polystyrene
CSV	Comma-separated values
D_{eff}	Effective Diffusivity coefficient
DLS	Dynamic light scattering
D_W	Theoretical diffusion coefficient of spherical particles in water
FCS	Fluorescence correlation spectroscopy
FEUP	Faculdade de Engenharia da Universidade do Porto
FOSS	Free and Open-Source Software
FRAP	Fluorescence recovery after photobleaching
GNU GPLv3	GNU General Public License v3.0
GUI	Graphical user interface
i3S	Instituto de Investigação e Inovação em Saúde
ISO	International Organization for Standardization
MEB	Mestrado em Engenharia Biomédica
MPT	Multiple Particle Tracking
MS	Microsoft
MSD	Mean-squared displacement
MVC	Model-view-controller
NPA	Nanoparticle Tracking Analysis
OS	Operating system
PBS	Phosphate buffered saline
PdI	Polydispersity index
PEG	Polyethylene glycol
PPG	Polypropylene glycol

List of symbols

α	Anomalous transport exponent
τ	Time scale

Chapter 1

Introduction

The main principles of Multiple Particle Tracking (MPT) as well as its most common applications will be introduced in this chapter. The importance of this kind of analysis is increasing as the use of nanoparticle in Healthcare has been growing.

1.1 Nanoparticles

Nanoparticles are typically defined as structures with at least one dimension in the nano-scale (nano refers to the metric unit of length, meaning for instance that 100 *nm* is equivalent to 10^{-7} m [1]). According to several publications, nano scale ranges from 1 to 100 *nm* are usually considered [2–4], although they agree that the higher limit of this range is just consensual and not definitive. They suggest that this 100 *nm* size limit is not precise and have no strong foundation.

Nanoparticles are useful in a variety of areas, from medicine and pharmaceuticals [5–9] to physics [10–12], from optics [13–15] to electronics [16–19], among others. The most recent and probably notorious success was the use of lipid nanoparticles in the development of mRNA-containing COVID-19 vaccines from Pfizer/BioNTech and Moderna [20]. In fact, according to the International Organization for Standardization (ISO) document ISO/TS 80004, its use history can be tracked to Greco-Roman times [21]. Even in healthcare, it has been long used since the early 16th century, according to the same document, by Paracelsus.

As for recent times, studies on nanoparticles have heavily increased (Figure 1.1). Over the last decade publications have almost tripled, from 12,040 publications in 2010 to 30,824 publications in 2020 (an increase of 2.6 times). And the researchers are keeping the pace. Over the first eight months of 2021, there were already 19,832 publications. From these numbers, it is possible to understand the importance of nanoparticles for science and for society, as the aim of those studies is most likely to bring benefits to all.

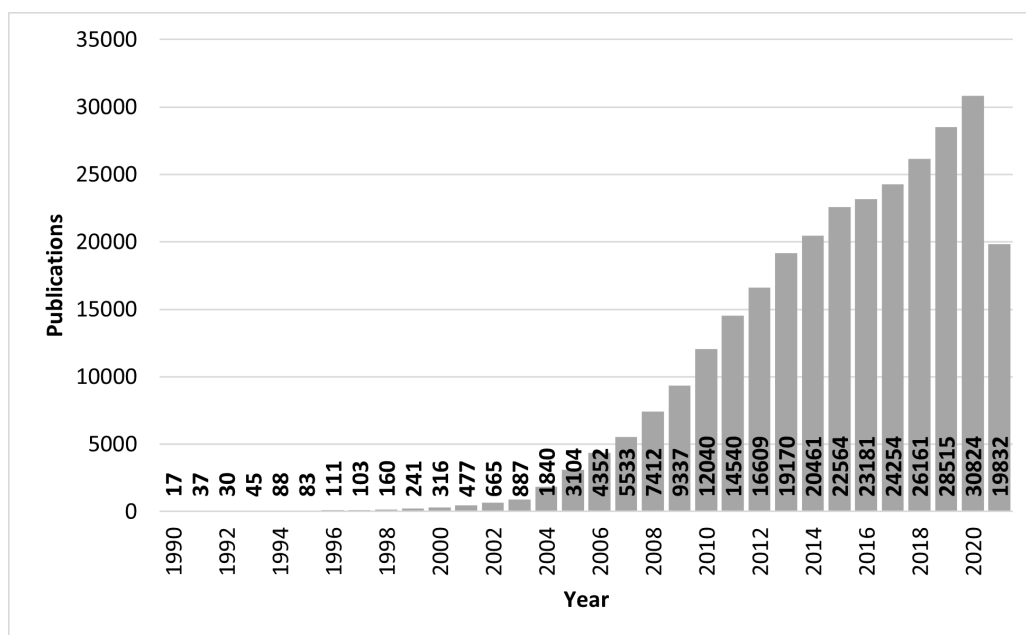


Figure 1.1: Publications containing the term 'nanoparticle' over the past 30 years (data collected from PubMed on August 20, 2021). Data for 2021 includes only the first 8 months.

1.2 Nanotechnology

As with nanoparticles, nanotechnology had great improvement along the past decades, with several contributions to scientific breakthroughs as well as everyday solutions reaching the global market [22]. Therefore, humans are more exposed to nanomaterials. This exposure can be intentional (as in the use of nanomedicines), or not (resulting from contact with nanopollutants), and the outcomes of such event depend on complex interaction in the human body, namely mucosal site of deposition, which need further studies [23]. For example, nanoparticle use depends mostly on their ability to transport across different natural barriers and media.

1.3 Particle tracking

Tracking a particle trajectory is important in order to verify the particle's ability to move in a particular medium of interest. One characteristic was defined in 1827 by the Scottish botanist Robert Brown [24]. According to his studies, the migration process of the particles from one point to another occurs due to concentration difference, and it is called diffusion. To verify the diffusivity, it is necessary to know the particle position over time. Video microscopy, for example, is able to record particle movement. The video capture is done frame by frame, as an image sequence, during a certain period of time and with a defined time lag (timescale). In fact, the research on this subject has seen tremendous attention over the last decade, as seen in Figure 1.2.

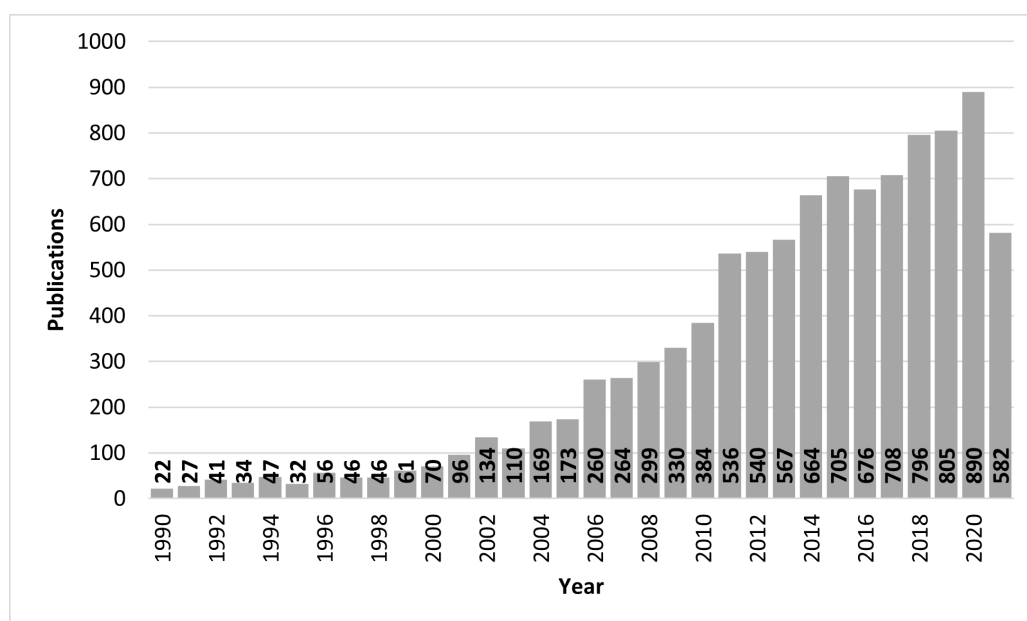


Figure 1.2: Publications containing the term 'particle tracking' over the past 30 years (data collected from PubMed on August 23, 2021). Data for 2021 includes only the first 8 months.

According to Pubmed, it is possible to observe a trend towards increased research output on the topic. For example, the number of publications increased from 384 in 2010 to 890 in 2020. Over the first eight months of 2021, there were already 582 publications on this subject.

Particle tracking methods usually rely on one of the following categories:

- Methods that measure the ensemble average dynamics of particles
 - Fluorescence recovery after photobleaching (FRAP)
 - Fluorescence correlation spectroscopy (FCS)
 - Dynamic light scattering (DLS)
- Methods that can discriminate single particle movement

Although ensemble methods can provide enhanced signal, heterogeneous particle populations or media, detail information is still overlooked [25]. MPT is a technique that enables tracing trajectories of individual particles, molecules, or other biological structures at a high spatiotemporal resolution in complex biological medium. This medium can be the intracellular environment or the extracellular matrix, as well as fluids such as blood or mucus [26].

The MPT technique has been used for several purposes in the biomedical field, for studies related to proteins, lipids and molecular motors [27], cell migration and mobility of pathogens [28, 29], mechanical properties of isolated nuclei in living cells [30], or microstructure and rheological profile of mucus and other viscoelastic fluids [31–33].

1.3.1 Difusivity

Diffusion is studied by analyzing particle's motion and how it compares with pure Brownian motion. Therefore, it is easy to understand the importance of diffusivity studies, as it verifies the particle's ability to move in a given medium. When considering a therapeutic nanosystem, diffusive behavior is typically considered beneficial, because it means the particle can freely move through a medium.

1.3.2 Mean-squared displacement

In order to verify the diffusivity, it is necessary to know the particle position over time. From video microscopy frames it is possible to get the particle position. But the mean displacement is not enough to get the particle motion, due to the random nature of Brownian motion [34]. The motion is calculated using mean-squared displacement (MSD) values.

1.4 Nanoparticle drug delivery

There are many studies on the use of nanoparticles as drug delivery carrier in order to more effectively achieve and prolong therapeutic concentrations at the site(s) of interest. As a result, this approach is typically expected to get less side effects and better action with lower doses. According to Pubmed, it is possible to observe a trend towards increased interest in the field over recent decades (Figure 1.3). Over the past decade it is possible to see that publications increased from 2,551 in 2010 to 6,727 in 2020. Over the first eight months of 2021, there were already 3,843 publications on this subject.

The development of nanomedicines may benefit from MPT, as it allows the understanding of the transport of nanocarriers in biological media and helps to design engineering strategies that can make it possible to overcome natural barriers to drug delivery and result in enhanced therapeutic outcomes [35–37]. Nevertheless, MPT analysis has its own limitations, in particular due to its two-dimensional tracking nature, resulting from rapid in-and-out movement of particles from the focal plane. Moreover, extrapolation to 3D movement is limited to locally isotropic media [38]. Data output can also be considered a limitation, since it is highly dependent on user input, which may compromise objective analysis.

1.5 Analysis of multiple particle tracking data: state of the art

Analysis of data generated from MPT experiments is usually done by computer algorithms. Although there are different tools available, there are also plenty of use cases. As expected, not every tool fits the needs of every use case. Conducting MPT experiments is simple and accessible [38]. Its processing cascade includes preparation of nanoparticle solution, video capture, video analysis and data analysis.

The nanoparticle solution typically uses fluorescently labeled molecules or structures in a biological media. Then, video is captured in a microscope with a high sensitivity camera. The microscope must be able to export video tracking data for trajectories analysis. The video analysis extracts particle frame-by-frame coordinates and defines particle trajectories. There are several image processing programs that can perform the video analysis, including microscope program, a third party program (usually including several plugins for different purposes) or even a specific algorithm [39, 40]. For example, the ImageJ/Fiji program

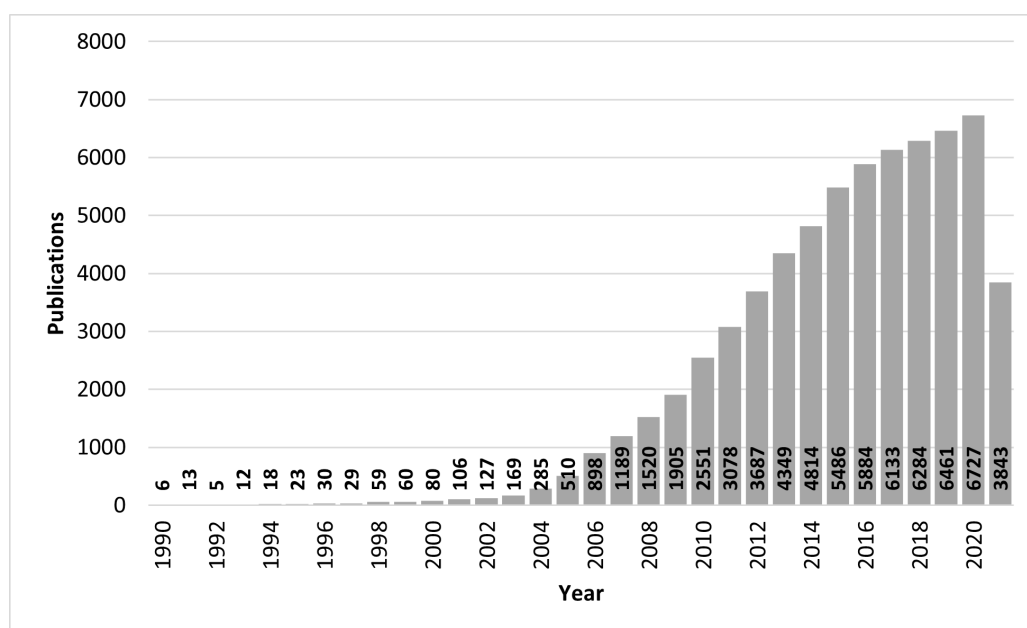


Figure 1.3: Publications on 'nanoparticle drug delivery' over the past 30 years (data collected from PubMed on August 23, 2021). Data for 2021 includes only the first 8 months.

is widely popular for image processing. It is based on the use of plugins, meaning it provides a base platform for developers to add (or improve) plugins. MosaicSuite [41], TrackMate [42] and NanoTrackJ [43] are a few examples of image processing plugins available for ImageJ/Fiji.

The video analysis provides trajectory data for all the particles it finds in the video, which means large datasets with more than 50 thousand lines of raw data. It is possible to extract quantitative information from these datasets, namely biologically relevant parameters that allow understanding the diffusive behavior of particles or characterizing the rheological properties of the specimen in which these are embedded. Although the motion analysis provides valuable particle information, manual processing of such large datasets can lead to errors and is extremely time-consuming [44]. To prevent manual handling, there are various useful software available for performing this task [45–48]. However, they offer limited possibilities for data processing, require a paid license, require advanced computing skills, or even rely on paid software (such as MATLAB). There are also some microscopes that have software for MPT analysis (e.g., NIKON NIS Elements or Malvern NTA software), although their use is limited to specific hardware [49, 50]. There are also similar open-source instrumentation software options available, but still with a limited number of compatible microscopes [51].

In short, there are currently several tools for particle tracking [52]. Nevertheless, there are still no tools able to provide free, easy-to-use, rapid analysis and editable reports in one single program. Since MPT analysis cascade is simple, being data analysis time-consuming and the procedure most prone to error, the protocol for MPT analysis should address mainly data analysis.

1.6 Final remarks and work objective

This chapter highlighted the increasing importance of nanoparticle use in the biomedical and medical fields. It also presented the techniques used for particle tracking studies and mentioned some of its uses. Of particular importance, it is important to establish a protocol to reduce the current limitations and drawbacks that exist in analyzing data generated from MPT experiments. This area still relies on sub-standard tools and complex protocols to get results that really matter to researchers.

Therefore, this work mostly details on the development of a user-friendly software that can provide a rapid and reliable analysis of particle trajectories extracted from MPT video microscopy.

Chapter 2

Materials and methods

To easily analyze particles in a specific medium, either of complex biological nature or other, it is common to define experimental protocols for researchers to use. Each protocol has its particularities, including used materials and software. It may use the microscope software not only for image acquisition, but for particle tracking and analysis as well. Some protocols may use proprietary software¹. Some use only Free and Open-Source Software (FOSS)². In some cases, both proprietary and FOSS may be used.

In this chapter, the main goals are to describe the reference protocol, and define what needs to be solved or improved.

2.1 Materials

For the nanoparticle solution, the following was purchased:

- Red fluorescent carboxylate-modified polystyrene (COOH-PS) nanoparticles with nominal mean diameter of 100 *nm* or 200 *nm*, from Molecular Probes (Eugene, OR, USA)
- Phosphate buffered saline (PBS) tablets and purified type II mucin from porcine stomach, from Sigma (St. Louis, MO, USA)
- 1.5 × 1.6 cm gene frames (65 μ L), from Fisher Scientific (Hampton, NH, USA)
- Amicon Ultra-0.5 mL (100 kDa MWCO) centrifugal filters, from Merck Millipore (Burlington, MA, USA)
- Poloxamer 407 – a poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) (PEG-PPG-PEG; average MW of 9.8 to 14.6 kDa) – was a kind offer from BASF (Ludwigshafen, Germany)

All other reagents and materials were of analytical grade or equivalent.

¹Proprietary software is a software that do not allow the access to its source code. Usually these are paid software and can be expensive.

²Free and Open Source Software is the software that allows anyone to use, copy, study and change the software in any way [53].

2.1.1 The reference protocol

As mentioned above, there may be multiple experimental protocols according to the microscope brand (and its software) and other aspects, as user knowledge of some other software. A protocol originally established by Crater and Carrier and previously used by our group at i3S was used as reference [8, 9]. The protocol can be used to analyze particle trajectories in complex biological mean such as mucus. It comprises a long list of steps that need to be conducted in order to accomplish the desired analysis. Briefly, those steps include:

1. Nanoparticle solutions preparation
2. Video acquisition using a microscope
3. Video analysis and report creation (using ImageJ [54–56])
4. Import report file into Microsoft (MS) Excel Excel
5. Manual finding of trajectories considered valid (with 560+ consecutive frames in a 575 total frames)
6. Manual copying of valid trajectory to a second MS Excel file, which contains adequate formulas to find the Mean Square Displacement (MSD) and the Effective Diffusivity coefficient (D_{eff}) for the current trajectory
7. Manual copying MSD and D_{eff} values to a third MS Excel file, which generates graphical displaying for MSD and D_{eff}
8. Repeat steps 5 to 7 for 100 valid trajectories
9. Manual copying data to a fourth MS Excel file to make logarithmic conversion of data
10. Manual copying data to a fifth MS Excel file that will allow particle characterization
11. Use a sixth MS Excel file to find the Einstein-Stokes diffusivity constant

Although the nanoparticle preparation is very straightforward, with not much to improve in terms of time efficiency or other aspects, there are some drawbacks in the data analysis steps conducted after the video microscopy. Therefore, the main problems are re-lated to steps 3-11, namely regarding accuracy, risk of data loss and time consumption.

2.1.2 Reference protocol revision

Even in a simplified form as shown before, it is possible to note the need to copy data over several MS Excel files. Because of this, the time to find valid trajectories and copy data for 100 trajectories is time consuming.

2.1.3 Data loss by user manipulation

The process of copying data is risky. The user may copy less data than the necessary or may paste it in the wrong cell or even delete formulas by mistake. This could lead to poor data output and time spent searching for erroneous data input.

2.1.4 Imprecise data from wrong formulas

Spreadsheet application may present errors in several ways, as presented by Panko [57], and that can be categorized as:

1. Quantitative and qualitative errors
2. Mechanical, omission, and logic errors
3. Mechanical errors
4. Logic errors
5. Omission errors
6. Errors by life cycle

There are several studies about Spreadsheet errors [58–62], as well as some news reports about it [63, 64].

2.1.5 Possible improvements to the reference protocol

Alongside other spreadsheet possible problems, the main problem has to deal with its time consuming nature. Additionally, the use of ImageJ and MS Excel (this last using six files) may be confusing. That is mainly due to the number of application instances (one of ImageJ and six of MS Excel). Working with that much opened files may be confusing. It gets even worse considering that each video gives about ten valid trajectories. Thus, the entire analysis process can last for hours. This is valuable time spent in tasks that could be automated by the current available technologies.

Since the current protocol needs improvement, different strategies were considered to address the main step of the process as well as expected difficulties and pros and cons. It is important to remember the four basic steps for the protocol to take place:

1. Nanoparticle solution preparation
2. Video acquisition
3. Video analysis
4. Data analysis

Although the aim of this work is mainly on steps 2 through step 4, the solution preparation was also reviewed.

2.1.6 Data analysis more efficient

Analysis of the video could be considered the most complex part of the whole process. That is due to the computational need to recognize the particles in each frame and relate each particle to its position on the next frame, defining what is called a trajectory (see Figure 2.1).

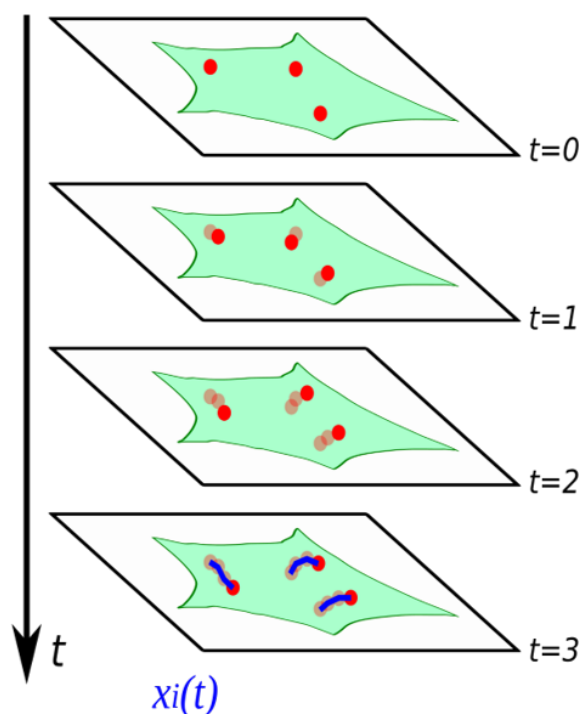


Figure 2.1: Frame by frame relation while detecting particles to define trajectories. Image from Wikimedia at <https://commons.wikimedia.org/wiki/File:Singleparticletracking.svg>, licensed under CC BY-SA license.

2.1.6.1 Improve ImageJ Mosaic plugin

As mentioned before, there are several tools in use for image and video analysis. One of those tools is ImageJ. This application has several tools for image analysis and processing, be it from its own capabilities or from plugins. One known plugin for video analysis is the Mosaic plugin, which can perform analysis on several video file formats and, as result, give data in an interchangeable format such as comma-separated values (CSV). Although the Mosaic plugin provides many functionalities, at the time of this work it was still not able to conduct trajectory analysis. Therefore, there is an opportunity to improve the plugin by adding such capability.

2.1.6.2 Develop an ImageJ plugin

Given that the Mosaic plugin allows changes, it is possible to make changes to it or request changes to be done. Either one of the options requires approval from the Mosaic development team. Therefore, as ImageJ plugin development can be done by anyone, it seems to be easier to manage a development process than engage in an ongoing development maintenance of others.

2.1.6.3 Develop a new analysis application

Integrate with existing tools and applications would make the entire analysis process more seamless, preventing the use of too many tools and avoiding complexity. Nevertheless, even seeming to be easier

to develop an ImageJ plugin than change the Mosaic plugin, both would need integration. A change to existing plugin requires integration with the plugin itself, while a new plugin requires integration with the ImageJ application. Considering the ability to control the overall behavior of the application, one of the options for data analysis is developing a new application, independent from ImageJ, but able to import output reports from ImageJ.

2.2 Improvement details

While trying to find the best possible conditions on each step of the protocol, a new protocol for multiple particle tracking video microscopy should be created for characterization of nanoparticle transport in complex biological environments. As previously mentioned, the main issue needing improvement is data analysis, which is directly related to video acquisition since a high-quality video is easier to analyze by image processing programs.

2.2.1 Defining video analysis approach

Video analysis involves loading the video file, identify particles in each frame, link particles from frame to frame (creating trajectories), and export trajectory data. The reference protocol uses ImageJ for this purpose. Although Mosaic Plugin performs in a satisfactory way, it is still very time consuming. Alongside, results are generated as basic data tables. Thus, it is not easy to get the desired data.

2.2.1.1 Improve ImageJ Mosaic plugin

The ImageJ Mosaic plugin does a great job in image segmentation and analysis but does not achieve the main aim of the study, which is to characterize particle behavior. It is developed in Java and allows anyone to propose changes. The author would need to study Java, the Mosaic plugin code in depth in order to develop the desired result outputs (see Table 2.1).

Table 2.1: Pros and cons of ImageJ Mosaic plugin.

Pros	Cons
Java is a cross platform language	Learning curve tends to be slow
Vast documentation available	Need to learn Java in depth
	Need to learn Mosaic plugin implementation

2.2.1.2 Develop an ImageJ plugin

Other options would be developing a new ImageJ plugin that meets the desired purpose of analysis. This would be assertive, but it would lead to more specific development processes. Similarly to the previous option, it would be developed in Java (see Table 2.2).

Table 2.2: Pros and cons of developing an ImageJ plugin.

Pros	Cons
Java is a cross platform language Vast documentation available	Learning curve tends to be slow Need to learn Java in depth

2.2.1.3 Develop an application

Development of an application from scratch seems to be the most suitable option. It would need to meet some requirements for best fitting the current trend of cross platform programs. In this option, the author would have to choose a programming language that needs to meet specified requirements. Still, there would be some pros and cons (see Table 2.3).

Table 2.3: Pros and cons of developing an application.

Pros	Cons
Author's preferred programming language Liberty to develop its own design patterns	Development needs to be from scratch

2.2.1.4 Defining the approach

Every option has its pros and cons and the time to develop each one of them needs to be considered in order to make the best choice for the current project. It may be focused on just a small part of the overall process, or the entire redesign of the tool in order to lead to a desired outcome. Given the freedom of a full development cycle, and despite the amount of programming work to be addressed, the chosen path was the development of a new application tool for the purpose of the new protocol.

There are currently several programming languages available for this purpose. It is desirable that the system works on different platforms (Windows, MacOS and Linux), so that it is possible to have a wider range in its use. After researching the potential of different programming languages, the following stood out:

- Java [65]: it is multi-platform and, in addition, it is used in the development of plugins for ImageJ [66]. It can be used for calculations [67] or for creating graphical user interfaces (GUI) [68]
- Python [69]: it is used in machine learning [70], deep learning, artificial intelligence and for analysis of scientific data (Data Science [71–74]). With some extensions, it can be used for GUI development
- MatLAB [75]: it is widely used in mathematical calculations and in the Engineering area [76]

From the programming languages listed above, it was decided to discard MatLAB, since it is proprietary (depends on purchase of license). The license cost is not desired as the intent is to make the program widely available and make it accessible for as many research groups as possible. Octave [77] is a free software and could replace the previous [78]. However, Python is also capable of performing the necessary processing and is also non-proprietary. Thus, Python was selected to perform the necessary calculations. Moreover, Python is getting great results in Data Science and Machine Learning that could be useful, and may allow future improvements in this project, for instance by adding Neural Network algorithms. Other

important aspect related to the programming language selection is its popularity, since it gives an idea of its future and, consequently, the maintainability of the application. According to Google Trends [79], Python is the most searched programming language among the options given above.

2.3 Software development and data processing

The developed software was named MPThub due to the intention to add more features in the future. It was developed to be simple, user-friendly and fast while enabling researcher to perform particle tracking analysis without compromising the reliability of the result. It has no requirements of prior programming knowledge and uses freely available software and packages such as Python (v. 3.9.5; Python Software Foundation, Wilmington, DE, USA, available at <https://www.python.org/>) as programming language due to its open access and open source status, reliability/stability, relatively fast run time and low memory consumption, reduced development time, and condensed amount of generated code as compared to other languages [80].

While Python comes with a large package library, other packages were further used to improve aspects of MPThub. The main packages used by MPThub are listed below:

- PySide6 (v. 6.1.1; for Graphical User Interface, available at <https://pypi.org/project/PySide6/>)
- Pandas (v. 1.2.4; for data manipulation, available at <https://pandas.pydata.org/>)
- Numpy (v. 1.20.3; for mathematical operations, available at <https://numpy.org/>)
- Matplotlib (v. 3.4.2; for plotting, available at <https://matplotlib.org/>)
- XlsxWriter (v. 1.4.3; for spreadsheet generation, available at <https://pypi.org/project/XlsxWriter/>)
- TrackPy (v. 0.5.0; for particle tracking analysis [81], available at <http://soft-matter.github.io/trackpy/v0.5.0/>)

The software was designed to fulfill the most notable need of the previous protocol, i.e. data analysis. In this sense, the aim of MPThub is to mitigate errors and data loss due to manual data handling using spreadsheets [60], and reduce data processing time comparing to the previous method [9]. To address these issues, MPThub comprises three main sequential tasks and one optional that is not always necessary:

1. Import data extracted from image processing programs
2. Analyze data
3. Export results
4. Adjust analysis configuration (optional)

MPThub is provided with default values for all required setting. Those values were defined during the experimental protocol elaboration but can be changed by the user. The setting values are stored in a local folder on the user's computer and can be changed according to the user's needs. These configurations are required to allow MPThub to understand the data and analysis parameters such as the relation between

metric units (nm) and digital units (px) for calculations regarding trajectories. Other information such as time lag, total frames and filter (minimal consecutive frames of a trajectory in order to be considered as valid) are also important during data analysis. There are also configurations used only during export, such as particle size, analysis time and temperature.

Users can interact with MPThub at different moments, for different purposes (Figure 2.2). It is important to point that some actions are dependent on others (e.g., 'Start Analysis' requires 'Import files' to be complete). Input files (.csv format) were generated from microscopy videos and included data for two-dimensional Cartesian coordinates per frame (see sub-section 2.4.3 for details on file acquisition and pre-processing).

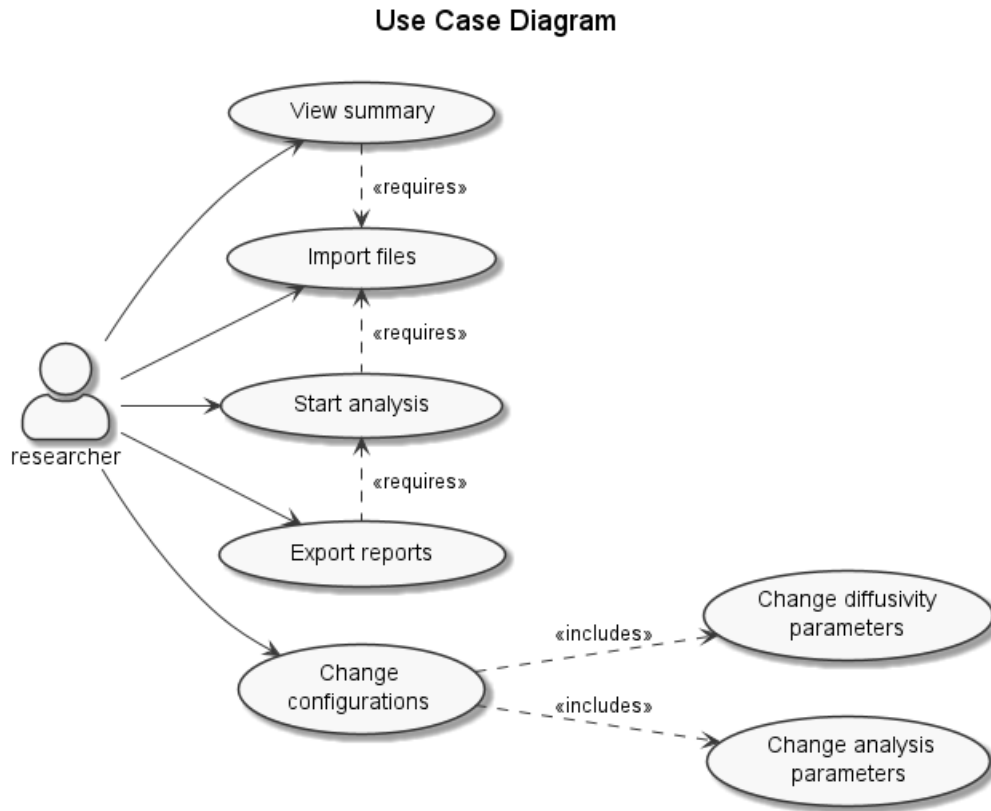


Figure 2.2: MPThub Use Case diagram.

Once the user has imported the files and there is trajectory information on summary, the 'Start analysis' option is enabled. Upon analysis, the software calculates:

1. Time-averaged mean square displacements (MSD or $\langle \Delta r^2(\tau) \rangle$)
2. Time-dependent diffusion coefficient or effective diffusivity (D_{eff})
3. D_W $\langle D_{\text{eff}} \rangle$ ratio
4. Anomalous transport exponent (α)

MSD values for individual trajectories were determined using the following equation:

$$MSD = [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2 \quad (2.1)$$

where x and y denote coordinates between consecutive τ intervals [38]. For a particle diffusing in a simple viscous liquid, the MSD was related to D_{eff} according to:

$$D_{\text{eff}} = \frac{MSD}{2n\tau} \quad (2.2)$$

where n refers to dimensionality ($n = 2$ in a two-dimensional system) [38]. Ensemble averages of MSD ($\langle MSD \rangle$) and D_{eff} ($\langle D_{\text{eff}} \rangle$) were further calculated and plotted against lag time. The $\langle D_{\text{eff}} \rangle$ at a given lag time can be compared with the theoretical diffusion coefficient of spherical particles in water (D_W), as calculated by the Stokes-Einstein equation:

$$D_W = \frac{k_B T}{6\pi\eta r} \quad (2.3)$$

where k_B is the Boltzmann's constant, T the temperature, η the fluid viscosity, and r the particle hydrodynamic radius. The ratio between D_W and D_{eff} provides a relative measure of transport impairment of particles in a specific medium as compared to water. In many cases the diffusion of particles follows a power law scaling that allows determining α according to:

$$MSD = 4D_0\tau^\alpha \quad (2.4)$$

where D_0 is the time-independent diffusion coefficient. The value of α can be used to classify particles mobility as immobile ($\alpha < 0.2$), sub-diffusive ($0.2 \leq \alpha < 0.9$), diffusive ($0.9 \leq \alpha < 1.2$), or active ($\alpha \geq 1.2$) [8, 9]. The bibliography is not definitive regarding these ranges and, thus, it is possible to change them in a configuration window. The classification as diffusive corresponds to Brownian motion as predicted by the Stokes-Einstein relation [38].

2.4 Application of MPTHub

MPTHub addresses data analysis of the protocol after initial video processing. For this work, the data come from videos capturing the motion of polystyrene nanoparticles in a defined medium. Also, mucus surrogates containing a mucin network were used throughout.

2.4.1 Processing of polystyrene nanoparticles

The process started by washing the fluorescent COOH-PS nanoparticles once with ultrapure water using centrifugal filters (4,300 rpm, 10 min) to remove traces of sodium azide. Then, nanoparticles were re-suspended in the same solvent at concentrations varying between 5 and 20 $\text{mg} \cdot \text{mL}^{-1}$, and dispersions were stored at 4 °C until further use. In the case of 100 nm nanoparticles, a further incubation in a 1% (w/v) polymer solution overnight was considered in order to achieve coating with poloxamer 407. Nanoparticles were then washed trice again before usage, as detailed above.

The hydrodynamic diameter, polydispersity index (Pdl) and zeta potential were determined by dynamic light scattering and laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Nanoparticles were diluted between 0.1 and 0.01 $mg \cdot mL^{-1}$ in 10 mM sodium chloride (pH 5.8) and measurements were carried out at 25 °C.

2.4.2 Preparation of mucus surrogates and incubation with nanoparticles

An intestinal mucus surrogate was prepared by modifying the reference protocol recipe [8]: mucin was reconstituted in isotonic PBS (pH 7.4) at 30 $mg \cdot mL^{-1}$ or 50 $mg \cdot mL^{-1}$ with the aid of a vortex mixer. The mucus simulant was left for 30 min at room temperature to equilibrate before 65 μL being gently transferred into a frame chamber mounted on a glass microscope slide. Two microliters of nanoparticles dispersed in PBS were top of the mucus surrogate and left to equilibrate for 1 h at room temperature after sealing the chamber with a coverslip. The final concentration of nanoparticles in the surrogate was $9.0 \times 10^{-6}\%$ (w/v) for 100 nm or $7.5 \times 10^{-5}\%$ (w/v) for 200 nm nanoparticles. The transport of fluorescent nanoparticles was assessed by analyzing their trajectories in either mucus surrogates or water.

2.4.3 Microscope configuration and video acquisition

Improvements on the image quality of video acquisition may be of huge importance. Image quality is directly related to video analysis. So, to get good video analysis, the image quality poses a relevant aspect.

Nowadays, the microscopes have great lens and can produce high-resolution, high-quality images. So, there is not much to improve in this matter besides the establishment of a protocol that guarantees good image acquisition.

When acquiring video from the microscope, there are several technical characteristics to take in consideration, such as:

1. Magnification (how close or far the microscope lens is from the sample)
2. Resolution (the smallest spatial interval measurable by the microscope)
3. Exposure (the amount of time each frame takes to be acquired)
4. Frames (the number of frames that ultimately compose the video)

MPT videos were recorded using a Hamamatsu ORCA-Flash4.0 digital CMOS camera (Hamamatsu, Japan) mounted on a Leica DMI6000 inverted epifluorescence microscope (Wetzlar, Germany) equipped with a 63 $\times$ /1.30 NA GLYC-immersion objective. Videos were collected with the LAS X software at a temporal resolution of 33 milliseconds and pixel resolution of 0.159 μm (512 $\times$ 512 pixels, 16-bit, 20 sec duration).

A tracking resolution of approximately 20 nm was determined by calculating displacements of nanoparticles immobilized in a strong adhesive (super glue). Acquired video files (.lif format) were pre-processed using ImageJ/Fiji (v. 2.1.0) by first converting them to grayscale 8-bit .tif time-lapses, followed by background subtraction (rolling ball radius 10 pixels) and application of a Median filter (radius 1 pixel). Videos with evident signs of photobleaching were excluded. Two-dimensional Cartesian coordinates of particle

centroids were detected at sub-pixel resolution using the MosaicSuite 2D/3D single-particle tracking plugin developed by Sbalzarini and Koumoutsakos [41]. Here, particle detection radius, cutoff and per/abs were set to 3, 0.01 and 0.1, respectively, link range was set to 2 frames and displacement to 10 pixels. Data were exported as .csv files and analyzed with the MPThub software. Trajectories with at least 100 consecutive frames were considered valid for transport analysis. Three independent experiments, each collecting the trajectories of a minimum of one hundred nanoparticles, were conducted for different conditions. These last were then analyzed using previous method (using regular spreadsheets) to act as reference for future result validation.

Chapter 3

Results and discussion

The establishment of the protocol aimed at making it easy for researchers to perform MPT analysis, representing a fast, reliable, and user-friendly process. In that regard, the biological part is usually the subject of study and is usually changed according to analysis results and specific needs. The data analysis represents a standard procedure despite experimental differences in each MPT study. Therefore, this chapter focuses on the features of the developed software, MPThub.

3.1 MPThub features and data usage

Since previous protocol relied heavily on the use of spreadsheets (leading to time-consuming and risky processing), it seemed reasonable to develop a software that provides a clean, easy-to-use, safe and fast option. This involved the establishment of a graphical user interface that allows the user to interact with the software in the most straightforward manner possible. Its repository and release pages are hosted, respectively, at <https://github.com/lassisg/mpthub> and <https://github.com/lassisg/mpthub/releases>. It is a standalone program and has no dependencies. Users just need to download the latest release, unpack the .zip file and double click the MPThub executable file. It was developed with the intention to contribute to scientific research and applications, with no restrictions as to its use. Therefore, its license is permissive, but requires the source code to remain open-source.

3.1.1 MPThub development

MPThub is a small software, developed in less than 2,000 lines of code. Its architecture is based on the Model-view-controller (MVC) design pattern [82] but not fully adhered to it, since the model and controller part are mixed. The main advantage of using MVC is the ability to change only one aspect of the software without compromising others (Figure 3.1). Therefore, the main purpose of the use of MVC was allowing the separation of the GUI aspect in such a way that would allow future version to promote other user interfaces. Even more, since different operating systems (OS) have different design guidelines, it is possible to separate design according to OS.

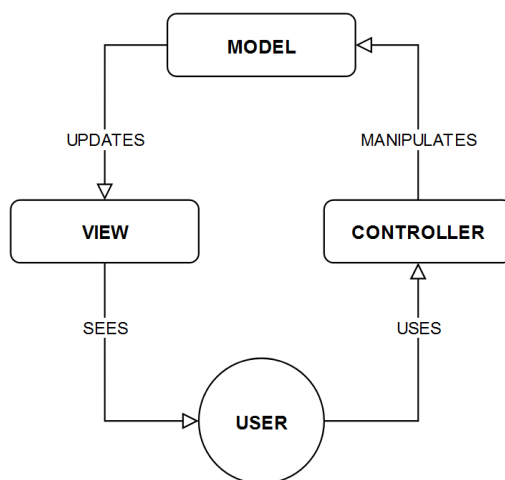


Figure 3.1: Diagram of MVC pattern interactions.

3.1.2 Graphical user interface

The main window of MPThub is divided in five areas: (1) menu bar, (2) toolbar, (3) summary, (4) summary totals, and (5) status bar (see Figure 3.2A-E). The most used tool buttons (import, analyze and export) have keyboard shortcuts (Ctrl+I, F5 and Ctrl+E, respectively) in order to aid users as they become more comfortable with its use.

MPThub was designed to be friendly yet flexible. It allows customization of configurations regarding analysis and diffusivity ranges (Figure 3.3A). Besides, it allows importing multiple files (.csv format) at once, which speeds up the process while making it even simpler by presenting a summary of the imported files immediately after importing. The summary presents basic information of total trajectories and valid trajectories per files. The valid trajectories value is defined according to the number of consecutive frames a particle is present, defined by the filter configuration (Figure 3.3B) according to the input of the user.

The user can add more files simply by repeating the import process and selecting different files, as well as remove files or clear the entire summary. All of the user actions can be executed with a single click on the desired option in the toolbar. Data analysis only requires a single command by clicking the 'Analysis' button. One should note that analysis is only allowed if there is data in the summary. Otherwise, the 'Analysis' button and menu option will be disabled. The results are exported in the same format as previously with improvements in formatting and use of spreadsheet functions to allow further analysis with spreadsheet functionalities, if needed (Appendix A, Figures A.1 to A.10).

There is no data storage besides configuration inputs, meaning that MPThub uses the computer memory to store and calculate all required values. This also prevents the need for database optimization and persistence. The software is portable and easy-to-use, requires no installation and uses less than 200 MB of disk storage (195 MB). The only extra space required is the configuration database, a SQLite database containing the configuration information. This database was chosen due to its portability and easiness of use, since it does not require extra installation and represents the creation of a single file [83].

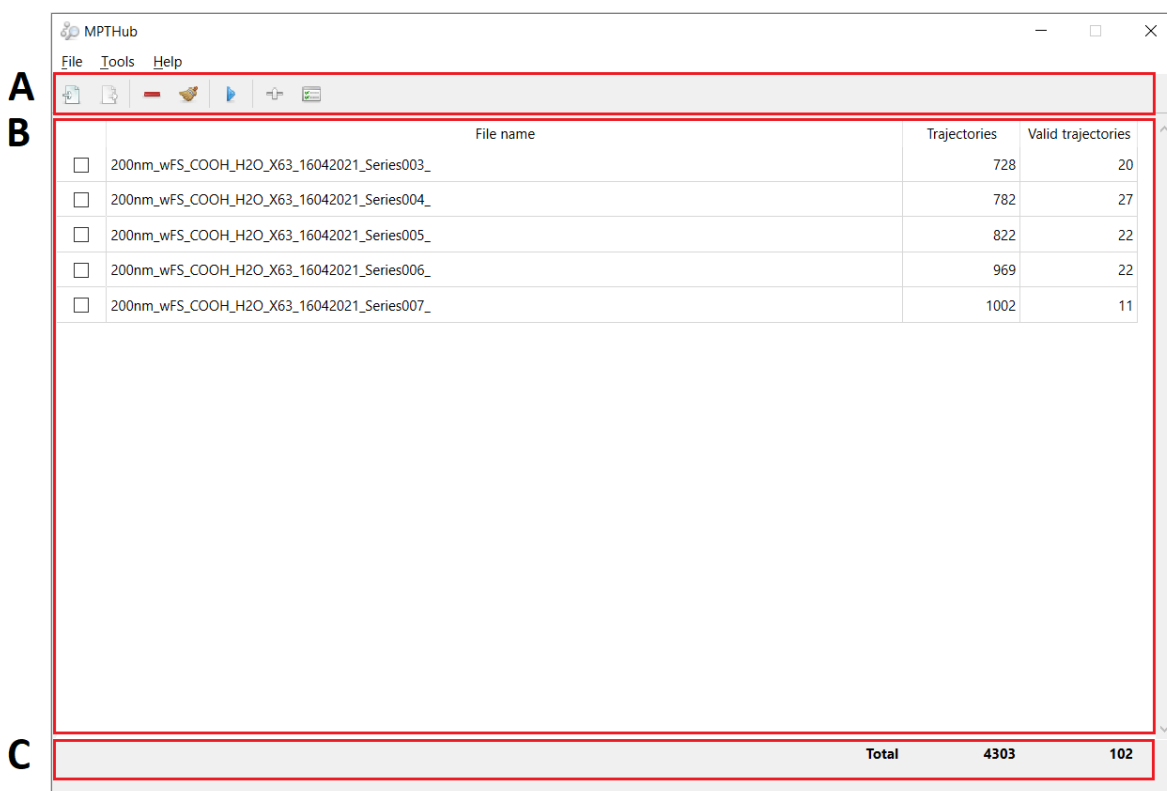


Figure 3.2: Main window of MPTHub divisions. **(A)** Menu bar; **(B)** Tool bar; **(C)** Summary; **(D)** Summary totals; and **(E)** Status bar. Summary data (file names, and total and valid trajectories) are shown after importing. All the icons used were designed by Yusuke Kamiyamane and licensed under CC-BY license.

3.1.3 MPTHub workflow

As mentioned in section 2.3, MPTHub comprises three main required tasks (1) import data, (2) analyze data, and (3) export results. Each one of these tasks can be considered a module as they have their own sets of processes (Figure 3.4).

3.1.3.1 Import data

To import data, the user either clicks the 'Import' button, or uses the menu option (File→Import) or the keyboard shortcut (Ctrl+I). Following user's selection, a selection window will prompt the user to select one or more files. This window opens at the last used folder location. It is imperative to select files (.csv) generated by MosaicSuite ImageJ Plugin. MPTHub only accepts data files in that specific format, which implies that future improvement may be necessary to make it more flexible regarding the importing of files from different origins such as other ImageJ plugins (TrackMate, NanotrackJ) or applications.

MPTHub will load the file and verifies if it complies with the expected format and content. In case the file does not match the specifications, it is ignored, and the next file is loaded. After all files are loaded, MPTHub will rename the data columns in accordance with TrackPy requirements. Once this is complete, the software will group the data by particle and count the number of groups (trajectories) and the number of

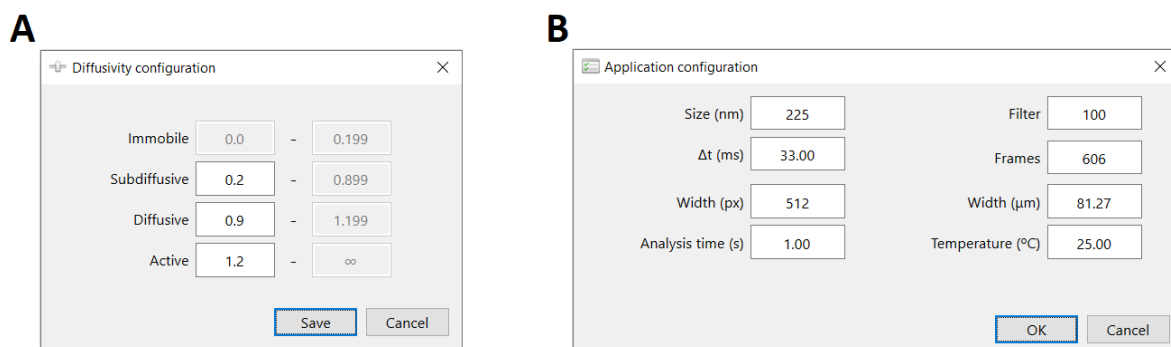


Figure 3.3: Input configuration parameters of MPThub. **(A)** Range for the anomalous diffusion exponent to define transport mode; and **(B)** Experimental conditions (particle size, temperature), input video properties (field width in pixels and microns, temporal resolution, total length in frames), and analysis parameters (filter - corresponding to the minimum number of consecutive frames needed to define valid trajectories, analysis time).

frames for each particle (frames per trajectory). If the number of frames per trajectory is equal or greater than the defined in the Filter configuration (Figure 3.3B), it is considered as a valid trajectory. Finally, MPThub builds and shows the summary with the data acquired from the files. A message is shown at the status bar informing the data was fetched.

3.1.3.2 Analyze data

In the previous task, MPThub imported data and found all valid trajectories. Those valid trajectories were saved in memory for future analysis. To start the analysis, the user selects the Tools→Start analysis menu or clicks on the 'Start analysis' button in the toolbar or can even use a keyboard shortcut (F5). This will load the TrackPy [81] algorithm to perform the calculations and receive the results.

3.1.3.3 Export results

Once the calculations were performed by TrackPy, the export menu is enabled and allows the user to export the results. To export these, the user can, once again, either click the tool bar button or select the menu option (File→Export) or use the keyboard shortcut (Ctrl+E). A system window will prompt the user to select a folder to save the report files. The folder selection window shows the last used folder. After selecting the output directory, MPThub will generate and save three reports: (1) Individual Particle Analysis (Appendix A, Figures A.1 to A.5); (2) Transport Mode Characterization (Appendix A, Figures A.6 to A.8); (3) Stokes-Einstein Calculations (D0_Dw) (Appendix A, Figures A.9 and A.10).

3.2 Validation of MPThub

The motion of fluorescent COOH-PS nanoparticles in intestinal mucus surrogates was analyzed in order to validate MPThub and demonstrate its potential. These particles were selected due to the substantial quanta emitted upon excitation, which enables the reduction of exposure time and therefore potential

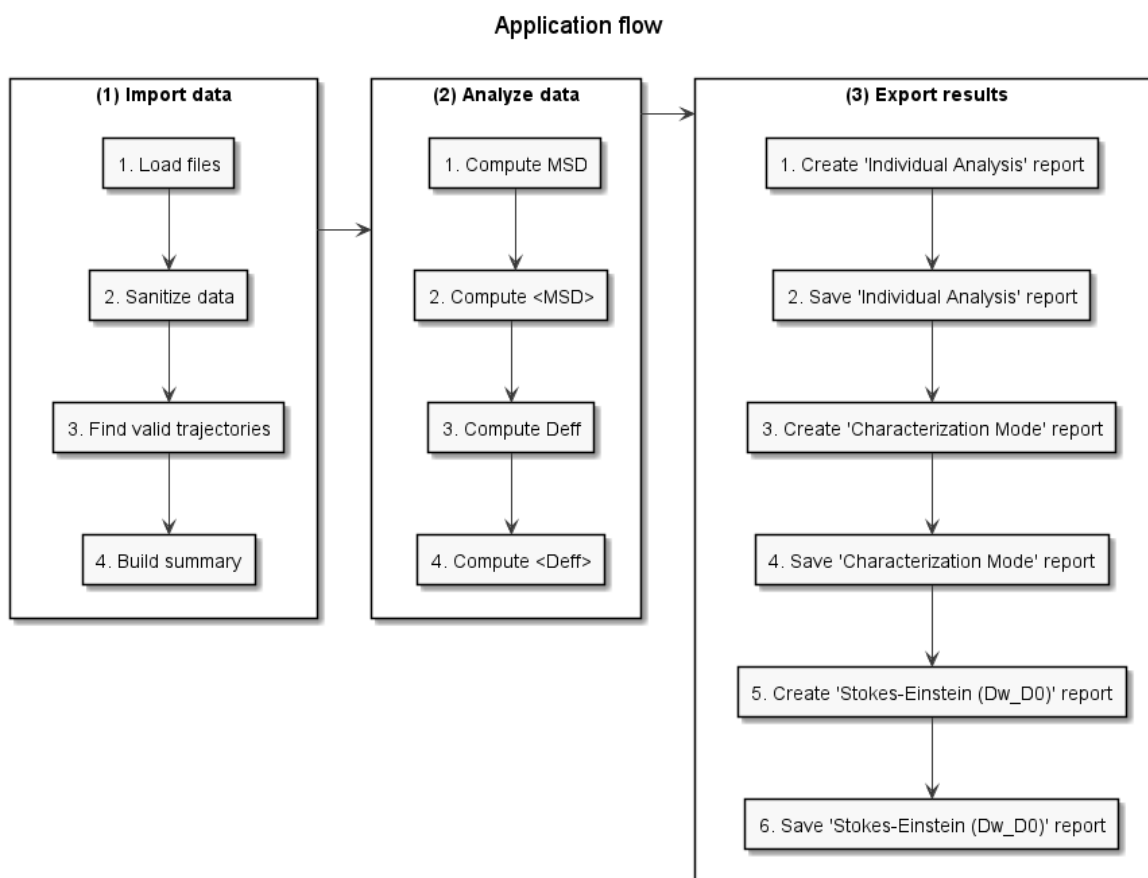


Figure 3.4: MPThub modules diagram flow.

dynamic errors. The nanoparticles were monodisperse and presented hydrodynamic values close to the nominal diameter provided by the supplier (Table 3.1). Changes in diameter for 100 nm nanoparticles modified with poloxamer were negligible, while the increase in zeta potential to near neutral values suggest extensive non-covalent coating with the triblock copolymer. The formation of a dense, low MW PEG corona is known to confer mucus-penetrating properties to similarly sized nanoparticles [84].

Table 3.1: Properties of COOH-PS nanoparticles. Results denote mean $\pm$ standard deviation ($n = 3$).

Nanoparticles	Coating	Hydrodynamic diameter (nm)	Pdl	Zeta potential (mV)
200 nm	–	225 ± 2	0.019 ± 0.011	-51.4 ± 1.8
100 nm	–	120 ± 2	0.102 ± 0.064	-42.6 ± 0.2
100 nm	Poloxamer 407	127 ± 4	0.036 ± 0.023	-4.6 ± 0.8

Using the developed software, the transport properties of nanoparticles in different media were analyzed. The first validation was made by comparing the motion of 200 nm COOH-PS nanoparticles in water and mucus surrogate containing 3% mucin (Figure 3.5A). The behavior of nanoparticles in water was random, which is typical of Brownian motion, and had practically constant spanned length. The transport of nanoparticles in surrogate mucus, in contrast, varied from highly restricted to nearly unhindered as

compared to water.

This implies a highly heterogeneous structure of the simulated medium, which is consonant with the properties of native mucus [85]. Ensemble (Figure 3.5B-C) and individual (Appendix B, Figures B.1 and B.2) analysis of MSD and D_{eff} confirmed and quantified the differences in nanoparticle transport of 200 nm COOH-PS nanoparticles. The gradual increase of $\langle \text{MSD} \rangle$ values with time scale, while $\langle D_{\text{eff}} \rangle$ values remained constant, suggest that particles experienced thermally driven Brownian motion [38]. Beside the variation aspects, $\langle \text{MSD} \rangle$ for 200 nm COOH-PS particles for $\tau = 1$ sec was almost 7-fold lower in mucus surrogate than for equivalent particles in water. At the same time scale, transport in simulated mucus was nearly 6-fold lower ($D_w/D_0 = 5.8$) than the expected in water, according to the Stokes-Einstein equation. Transport in water indicates an overall good agreement with theoretical diffusivity ($D_w/D_0 = 0.9$), with the deviation probably resulting from MSD variation at longer times scales [86].

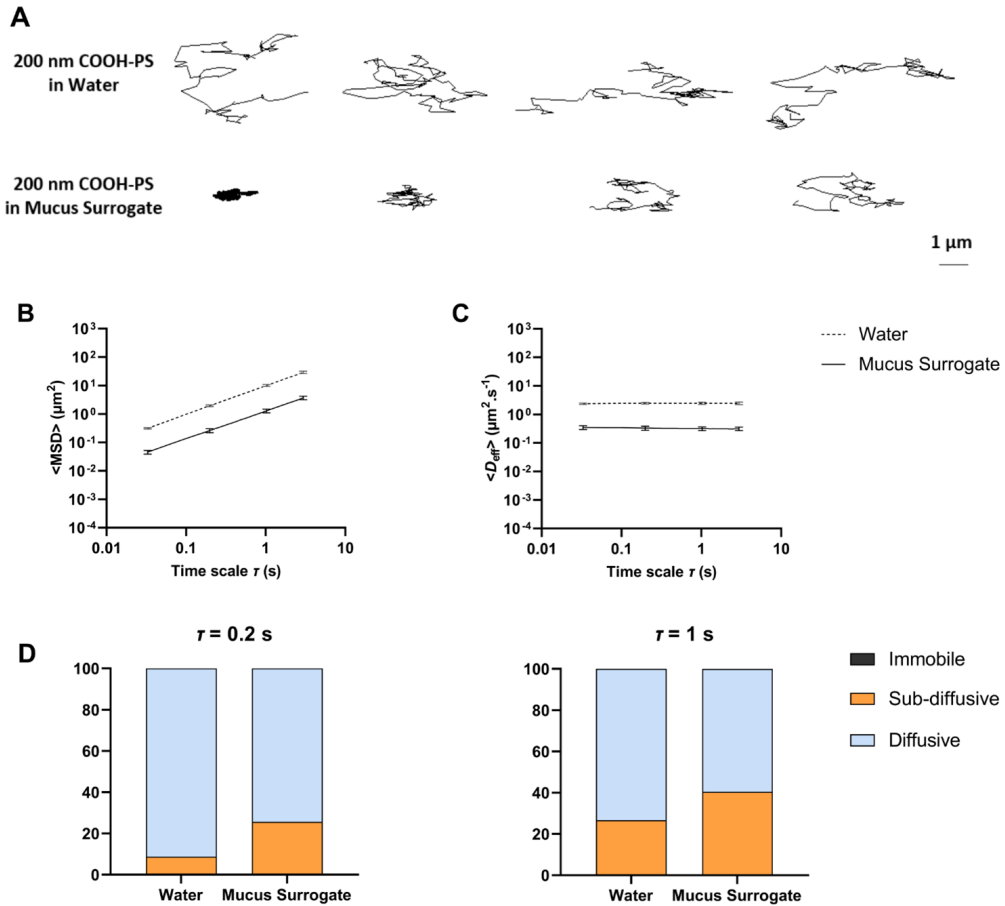


Figure 3.5: Transport behavior of 200 nm COOH-PS nanoparticles in water or mucus surrogate containing 3% mucin. **(A)** Representative trajectories for a total duration of 3 sec. **(B)** Ensemble-averaged geometric MSD and **(C)** Ensemble D_{eff} of 200 nm COOH-PS nanoparticles in water or mucus surrogate containing 3% mucin as a function of time scale. Data presented as mean $\pm$ standard deviation ($n = 3$). **(D)** Distribution of 200 nm COOH-PS nanoparticles in water and mucus surrogate (3% mucin) according to transport mode for $\tau = 0.2$ sec and $\tau = 1$ sec (data presented as mean values; $n = 3$).

The values obtained for 200 nm COOH-PS nanoparticles in mucus surrogate are in consonance with the values reported by Crater and Carrier [8] using similar experimental conditions, but at a longer time scale value (10 sec). Nevertheless, transport hindrance of 200 nm COOH-PS nanoparticles in either cervicovaginal or respiratory native mucus has been shown higher (at least three orders of magnitude when compared to the theoretical mobility in water [87, 88]) than in the present work. This observation seems to be justified by the structural differences between native and surrogate media (obtained by simply reconstituting mucin in PBS) [8]. Still, there could be other explanations for the higher mobility, such as the use of a short time scale, which allowed faster particles to contribute with multiple trajectories (due to their easiness to offset the focus plane of the microscope during video capture) [89], or the high ionic strength of the mucus surrogate that may represent a decrease in the effective surface charge of the nanoparticles and thus reduce electrostatic interactions with mucin [90].

For classifying the ensemble transport of 200 nm COOH-PS nanoparticles in water, average values for the anomalous diffusion exponent resulted in a typical pure Brownian motion in viscous fluids ($\alpha = 0.98$). In opposition, the mucus surrogate was marginally sub-diffusive ($\alpha = 0.88$) - probably due to the establishment of mild adhesive interactions with mucin and/or increased medium viscosity [87]. However, this value is within the range of similarly sized mucus-penetrating particles in native mucus [91].

The individual analysis data also allowed the COOH-PS nanoparticles classification in different classes of transport mode (Figure 3.5D). There were practically no immobile particles in either media, being nearly 90% of COOH-PS nanoparticles diffusive in water ($\tau = 0.2$ sec), while roughly 70% in mucus surrogate. The number of diffusive particles decreased for $\tau = 1$, particularly in water, which has been previously justified by the increase in statistical uncertainty for higher values of lag time, due to the calculation of its average consider less contributions of MSDs [50]. This effect was most evident in water since $\tau = 1$ represents more than 30% of the total duration of the trajectories.

To explore the applicability of MPTHub, further tests were made with different particle diameter (100 nm), surface properties (PEG-modified using poloxamer 407 as coating agent) and medium composition (5% mucin) of fluorescent nanoparticles. The result data is summarized in Table 3.2 and the ensemble and individual distribution of MSD and D_{eff} are detailed in Appendix B (Figures B.3 to B.6).

To explore the applicability of MPTHub, further tests were made with different particle diameter (100 nm), surface properties (PEG-modified using poloxamer 407 as coating agent) and medium composition (5% mucin) of fluorescent nanoparticles. The result data is summarized in Table 3.2 and the ensemble and individual distribution of MSD and D_{eff} are detailed in Appendix C (Figures B.3 to B.6).

Table 3.2: Transport characterization of different 100 nm COOH-PS nanoparticles in mucus surrogates containing either 3% or 5% mucin. Results are presented as mean values.

Coating	Mucin content (w/w %)	D_w/D_0	α
-	3%	6.0	0.91
-	5%	12.1	0.86
Poloxamer 407	3%	1.7	0.98
Poloxamer 407	5%	4.9	0.88

According to the data, transport of 100 nm COOH-PS nanoparticles in mucus surrogate containing 3% mucin was below the expected, but still within the diffusive range ($\alpha = 0.91$). When increasing the mucin content in the medium from 3% to 5%, the hindrance increased by about 2-fold, making the behavior

of particles marginally under diffusive. Still, non-covalent modification of the 100 nm PEG of COOH-PS nanoparticles affected mobility in both mucus surrogates, showing improved transport at 3% and 5% mucin that is closer to the predicted behavior of similar sized particles in water. These results are in line with the multiple observations that densely PEG-modified nanoparticles are able to present mucus-penetrating properties [92], even if, at higher mucin concentration, the anomalous diffusion exponent was virtually identical for modified and non-modified nanoparticles at 5% mucin.

The results from MPThHub analysis, when compared with the results from the reference protocol [8], were almost always identical. The differences occurred in specific scenarios when a particle trajectory was missing the coordinates of one frame. This is allowed according to the image processing software when linking particles frame-by-frame. As previously explained, due to the in-and-out movement of particles from the focal plane, a particle can go out of focus during one frame, resulting in a missing coordinate data. Here lies a difference in the calculation method. While the reference protocol ignored the missing frame data, TrackPy (used in MPThHub) does not. This affects the results from equation 2.1 calculations, resulting in different MSD and D_{eff} values. However, in addition to being considered a solution to an undetected issue on the previous protocol, the difference found did not affect the characterization results remained.

3.3 MPThHub performance

There is no objective procedure to estimate the time to run the previous protocol that relied on manual handling of data during analysis, as it heavily relied on the computer skills of the user and speed in copying, alternating windows and pasting data between spreadsheets [8, 9]. Importantly, individual copying and pasting of each trajectory from each ImageJ result file (.csv) was required. MPThHub analysis time was estimated at near 0.2 seconds, with import and export taking about 5 seconds each. There could still be the need to review analysis configuration, which would take extra time. Therefore, apart from the configuration review, the total analysis time using MPThHub is around 10 seconds. This time window of a few seconds compares to several hours typically required for conducting spreadsheet-assisted manual data processing. As for computer resources usage, some comparisons were made using different data inputs, with 2 and 4 files respectively. For each input group, we set different configurations, as shown in Table 3.3.

Table 3.3: Performance test configuration.

	Valid trajectories		
Input with 2 files	100	500	1,100
Input with 4 files	100	500	1,100

All the tests were conducted in 3 different computers with the following configurations:

- Computer 1: 11th Gen Intel® Core™ i7-1165G7 processor (12M Cache, 2.80 GHz), 16 GB of memory
- Computer 2: 4th Gen Intel® Core™ i7-4500U processor (4M Cache, 2.40 GHz), 16 GB of memory
- Computer 3: 7th Gen Intel® Core™ i3-7020U processor (3M Cache, 2.30 GHz), 4 GB of memory

The conducted analysis on MPThHub performance allowed to understand the impact of hardware and data volume in both time and memory use during import, data analysis and export.

3.3.1 Hardware impact on time

Each computer has its specifications, and it might interfere in the use of MPThub. Processor speed and memory capacity might interfere in time and ability to store data. The import process loads data into the computer memory. Therefore, more time should be needed when more data is loaded. As shown in Figure 3.6, since computer 1 has a faster processor, it loads data faster when compared to older computers.

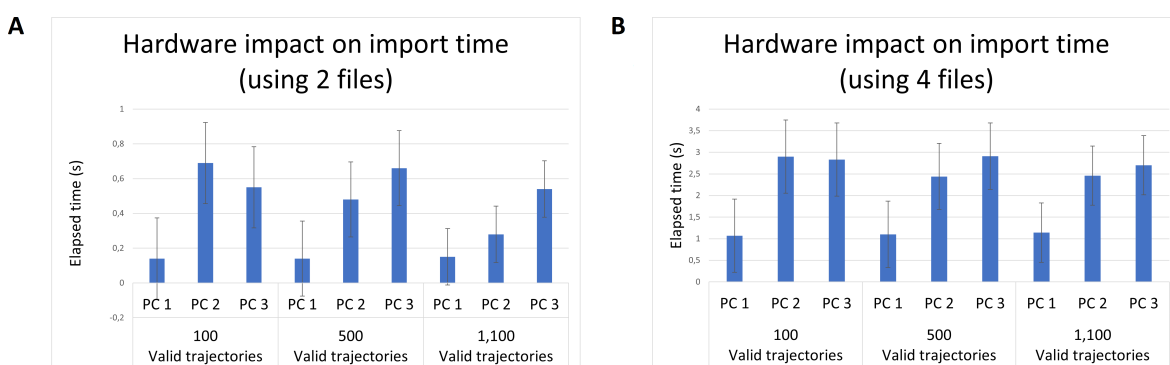


Figure 3.6: Hardware impact on import time. **(A)** Elapsed time to import 2 files; and **(B)** Elapsed time to import 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

Once the data is loaded into memory, the analysis is performed by TrackPy algorithm, which uses in-memory data provided by MPThub. Once again, the processing time is affected by the hardware (Figure 3.7).

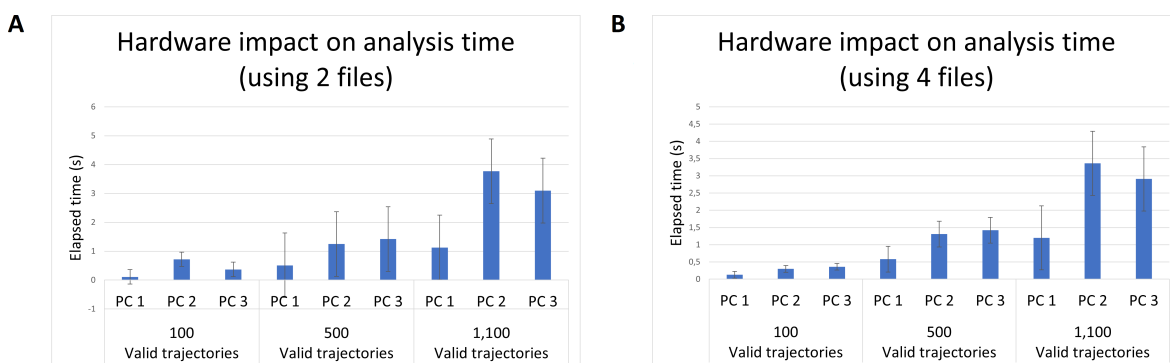


Figure 3.7: Hardware impact on analysis time. **(A)** Elapsed time to analyze data from 2 files; and **(B)** Elapsed time to analyze data from 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

The results export uses the computed values, added to the computer's memory, to generate editable spreadsheets reports. As all previous performance analysis, processor makes difference in time (Figure 3.8).

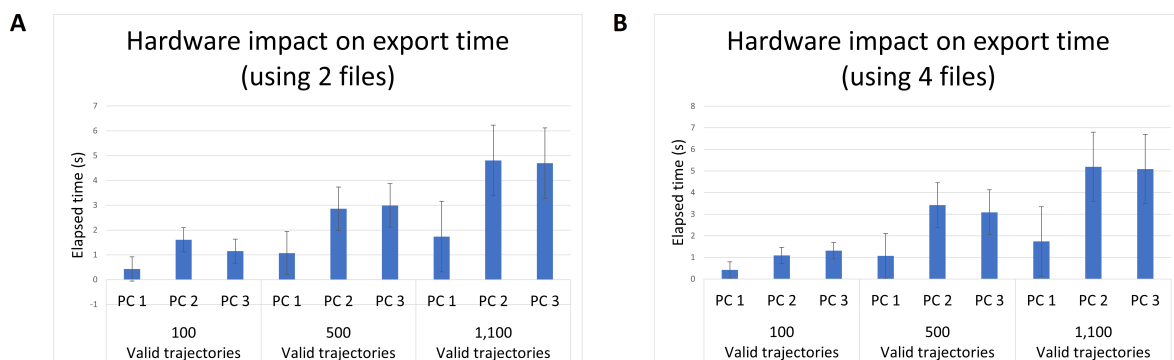


Figure 3.8: Hardware impact on export time. **(A)** Elapsed time to export data from 2 files; and **(B)** Elapsed time to export data from 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

3.3.2 Hardware impact on memory use

Since MPThub does not store any analysis value in databases, it is expected an increase of memory use as stored data grows along MPThub use. That is, starting from 87 MB of memory use (the memory used by MPThub before any interaction with the user), the memory use should be higher after import. Then the memory use should grow after analysis, since analysis data is stored. And more data is stored when exporting data, due to spreadsheet configuration values also stored in memory.

The hardware impact in memory use shown in Figures 3.9, 3.10 and 3.11, illustrates that the hardware differences had no relevant effect on memory use.

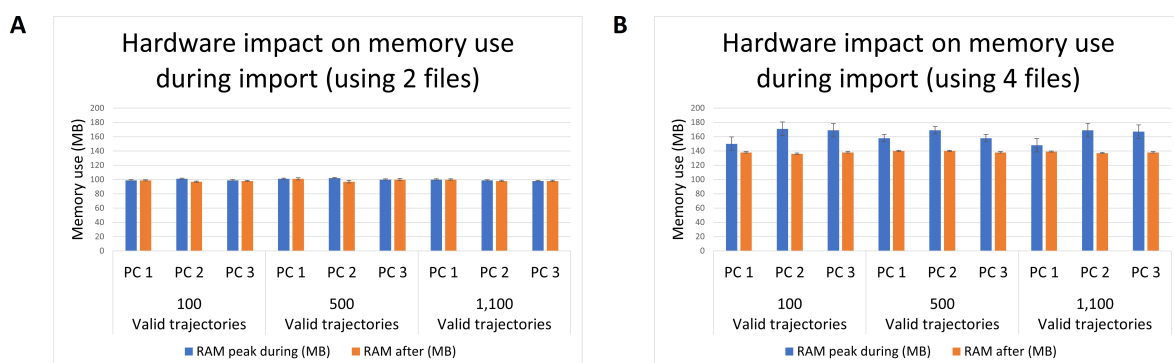


Figure 3.9: Hardware impact on memory use during import. **(A)** Memory use when importing 2 files; and **(B)** Memory use when importing 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

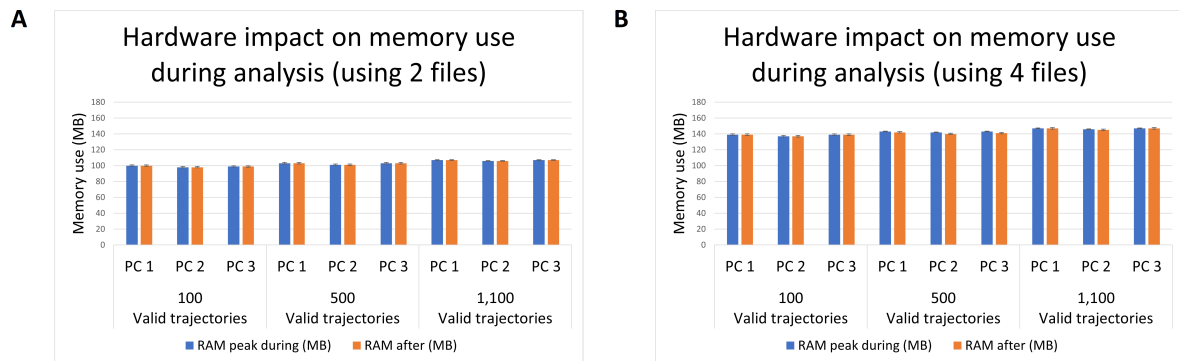


Figure 3.10: Hardware impact on memory use during analysis. **(A)** Memory use when analyzing 2 files; and **(B)** Memory use when analyzing 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

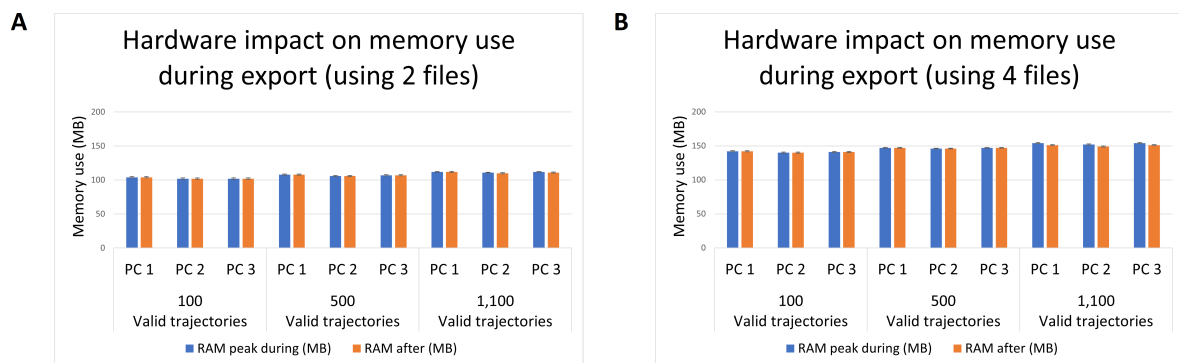


Figure 3.11: Hardware impact on memory use during export. **(A)** Memory use when exporting analysis data from 2 files; and **(B)** Memory use when exporting analysis data from 4 files. Results are presented as mean $\pm$ standard deviation ($n = 3$).

3.3.3 Data volume impact on time

Data volume impact was also tested, as data may vary according to each research needs. Both the input data and the valid trajectory data play important roles in understanding MPTHub behavior. Results from Figure 3.12 shows the impact of total data volume on import time. However, for import, the total valid trajectories is not relevant, as all the input data will be loaded into memory.

As for analysis process, there is no significant impact on time related to total data volume. Nevertheless, total valid trajectories affect processing time (Figure 3.13).

As occurred for analysis, the data volume effect on export is similar. The total data volume has no relevant impact, while valid trajectories have (Figure 3.14).

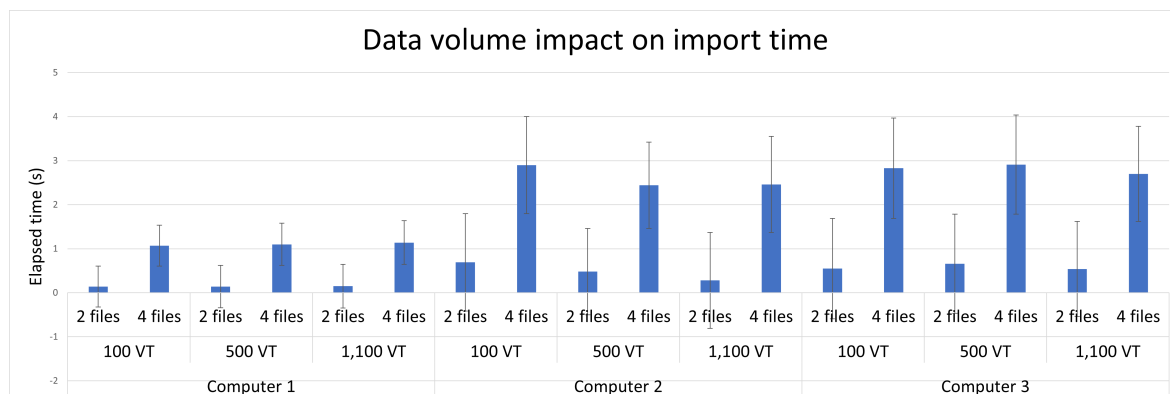


Figure 3.12: Data volume impact on time during import. Results are presented as mean $\pm$ standard deviation ($n = 3$).

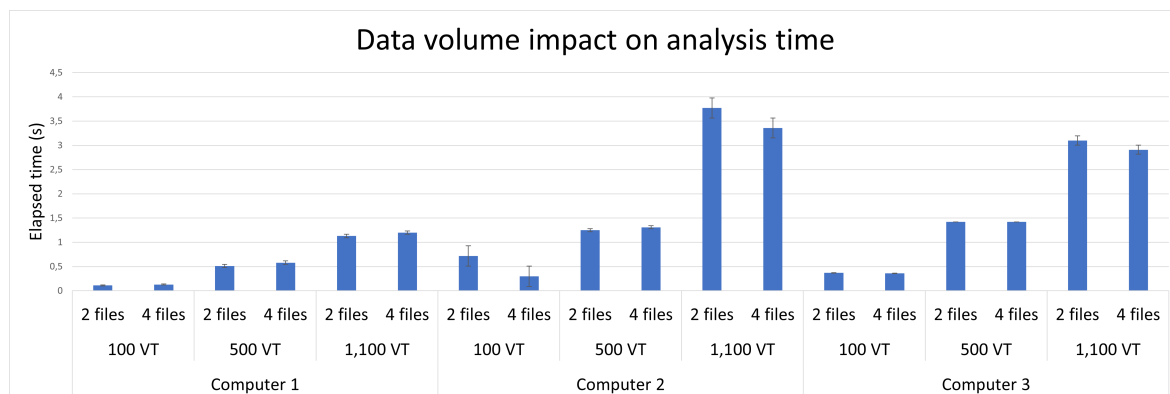


Figure 3.13: Data volume impact on time during analysis. Results are presented as mean $\pm$ standard deviation ($n = 3$).

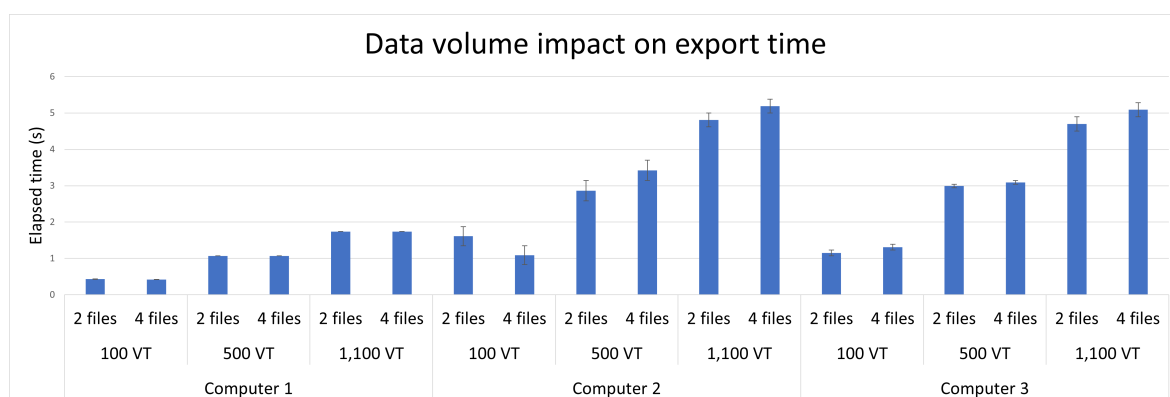


Figure 3.14: Data volume impact on time during export. Results are presented as mean $\pm$ standard deviation ($n = 3$).

3.3.4 Data volume impact on memory use

Since MPTHub does not use database for storing analysis values, increasing the amount of imported data leads to increased storage in the hardware memory. Continuous use of MPTHub generates more calculations and reports, which means more memory usage.

When analyzing memory use during input data import, MPTHub is affected by total data volume, which is stored in memory. At this stage, valid trajectories have no relevant impact on memory use (Figure 3.15).

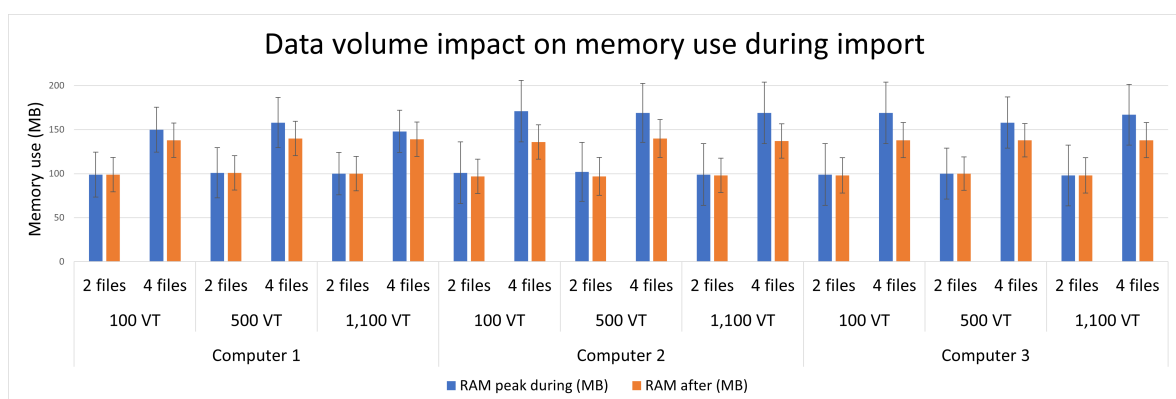


Figure 3.15: Data volume impact on memory use during import. Results are presented as mean $\pm$ standard deviation ($n = 3$).

Once again, the results confirm the expected behavior, showing an increase in memory use as the amount of data increases. Both analysis and export processes memory use increase with the data load. From Figures 3.16 and 3.17, for each valid trajectories group, it might seem like the total data volume cause impact on analysis and export memory use, but it is not. From Figure 3.15 we learnt that total data volume increases MPTHub memory use (4 files content uses more memory than 2 files content).

To better explain, let us take computer 1 as an example. It uses 100 MB of memory after importing 2 files data, while the data from 4 files takes 140 MB of memory. After analysis of 100 valid trajectories, computer 1 keeps the same memory use as before, almost 110 MB of memory use for data from 2 files and approximately 150 MB memory use from 4 files data. When exporting 100 valid trajectories data from 2 files, MPTHub increases memory use from 100 MB to 110 MB (10 MB of memory increase). When exporting 100 valid trajectories data from 4 files, MPTHub increases memory use from 140 MB to 150 MB (10 MB of memory increase).

Therefore, total data volume has no effect on MPTHub memory use during analysis and export, but only on import. As for the total valid trajectories, they cause impact on memory use during analysis and export, but do not affect import.

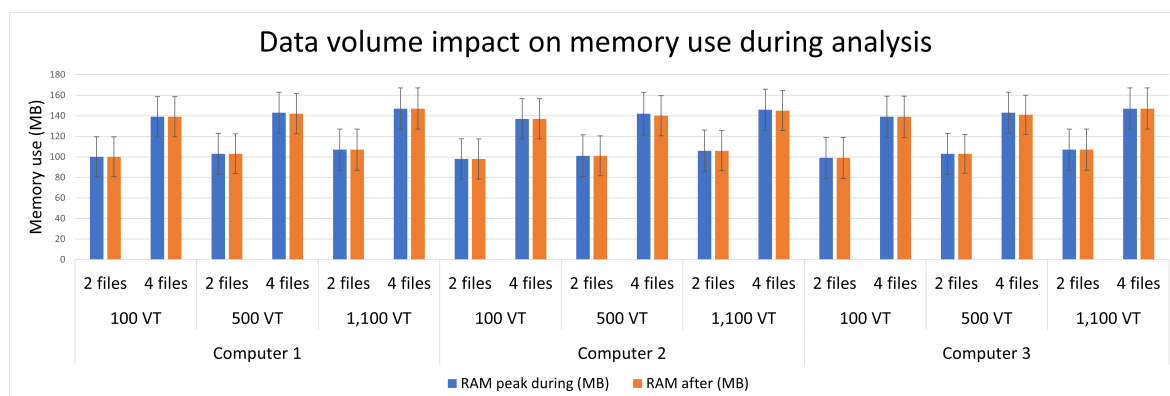


Figure 3.16: Data volume impact on memory use during analysis. Results are presented as mean $\pm$ standard deviation ($n = 3$).

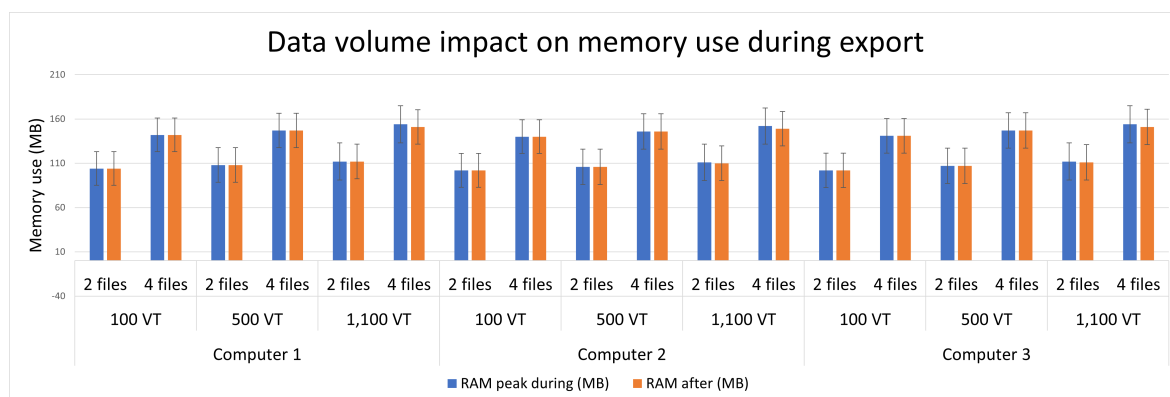


Figure 3.17: Data volume impact on memory use during export. Results are presented as mean $\pm$ standard deviation ($n = 3$).

3.3.5 Overall MPThub performance

When MPThub is open, before any interaction from the user, it uses approximately 87 MB of memory. Although the memory use increases with increasing number of valid trajectories, it is only for numbers greater than 1,000 that the memory consumption reaches almost the double of its idle consumption, reaching over 150 MB usage. However, most analysis are done with about 100 valid trajectories. Alternatively, the user can restart the software if memory issues occur. Regarding time performance, MPThub proved to give speed to the analysis process and, even on the less resourceful computer, with exaggerating 1,100 valid trajectories data, it took less than 6 seconds to perform the slowest task of exporting reports.

Overall, the MPThub was able to dramatically improve the previous MPT protocol used in our group at i3S, namely by reducing the amount of time and computer resources required. Results appear to support the software accuracy, speed, usability, and relevance. Even when used under extreme and unreal conditions, memory consumption by the MPThub was within reasonable values, and did not lead to system crashing or freezing in any occasion.

Chapter 4

Conclusions and future work

An improved protocol for performing MPT analysis in complex biological media, namely simulated mucus was established based on previous work developed by our group [9] and others [8]. Changes were particularly relevant in terms of data analysis as a result of the development of a new software for that purpose named MPThub. The complete protocol is detailed in Appendix C.

The developed software represents a new alternative for particle tracking analysis, providing an easy-to-use, free and open-source, lightweight graphical program. It rapidly imports raw tracking data from image processing software, performs analysis and exports results in less than one minute, which represents substantial timesaving when compared with previously used protocols involving spreadsheet-assisted manual handling of data. The output consists in well formatted spreadsheets containing objective parameters, charts and formulas that allow individual and ensemble transport characterization of fluorescent particles in biorelevant media such as mucus surrogates. This allows the user to further customize graphical presentation of generated data according to specific needs.

Data from 'wet lab' experiments proved helpful in showing the potential of the software. The most notable outcome was the reduced amount of time required for analysis. However, the potential of errors stemming from erroneous data handling were also removed since there is no need to manipulate raw data. High-throughput studies, in particular, may benefit from these features.

MPThub was developed under git version control and is hosted on Github as an Open-Source software, under GNU General Public License v3.0 (GNU GPLv3). This means that any user can contribute with bug report, bug fixes, feature request, improvements on code, or documentation. Moreover, it allows the creation of other software based on it while giving proper credit to contributors. MPThub was developed using Python programming language and is currently only available for Windows x64.

The current state of MPThub presents its first version and, naturally, there is still room for upgrades (e.g., to fix eventual issues, provide more functionalities, or improve any part of its code), some of which are already being enforced. Future work include (1) improving the compatibility with other image processing programs data (e.g., TrackMate, NanoTrackJ); (2) considering direct trajectory extraction from videos, which could minimize bias introduced by user input [44]; (3) exploring TrackPy resources, such as image analysis (this is a long-term addition, since it will require extra effort to perform additional tests and benchmarking); (4) improving the user interface 'aesthetics' (e.g., include graphical logos or improve 'brand' recognition); (5) adding data preview prior to export; (6) including more custom configuration possibilities

for the exported charts (e.g., chart themes); (7) adding the ability to accept custom report configuration (e.g., report name, suppress charts); and, finally, (8) considering the use of Artificial Intelligence (AI). Although currently available MPT methodology is highly used and proven ease, fast and reliable to perform, AI is growing fast and there are some studies describing the use of Neural Networks for particle tracking [93–96]. Finally, as the software becomes freely available online and is potentially used by others, feedback received from colleagues involved in the field may provide additional experimental validation and new hints as to future improvements of the MPThub.

Appendix A

Data reports spreadsheets

Three different reports are generated as result from MPThub use in a well-known and user-friendly spreadsheet file format (.xls), as shown in Figures A.1 to A.10.

	A	B	C	D	E	F	G	
1								
2	Timescale (τ) (s)	MSD 1 (μm ²)	MSD 2 (μm ²)	MSD 3 (μm ²)	MSD 4 (μm ²)	MSD 5 (μm ²)	MSD 6 (μm ²)	MSD
3	0,033	0,3595162	0,335247965	0,358997906	0,321676024	0,333997556	0,346448755	0,324
4	0,066	0,748341197	0,711585943	0,805129822	0,663219512	0,739098928	0,696488509	0,700
5	0,099	1,120268097	1,174051432	1,304841505	0,921620463	1,121483404	1,091246289	1,082
6	0,132	1,469545014	1,622128414	1,852732019	1,169742825	1,502705168	1,509443649	1,435
7	0,165	1,766896682	2,115902296	2,444736938	1,445253865	1,923771135	1,929793572	1,81
8	0,198	2,051934254	2,575221646	3,10054382	1,741620877	2,342187519	2,372116057	2,145
9	0,231	2,386773102	3,061268762	3,822138155	2,060132619	2,830352796	2,805928233	2,534
10	0,264	2,658738134	3,526845485	4,650600635	2,367288709	3,340736842	3,214870477	2,95
11	0,297	2,958861547	4,01410966	5,510904781	2,69034476	3,831873943	3,625852242	3,291
12	0,33	3,247820116	4,489055587	6,452327422	3,045685978	4,386212756	4,001671855	3,587
13	0,363	3,581053401	4,951982046	7,454170951	3,409032259	4,904573731	4,363976118	3,847
14	0,396	3,899347909	5,388378578	8,51775556	3,806257712	5,429963842	4,739145238	4,128
15	0,429	4,227369462	5,815310817	9,589483376	4,188656076	6,008060872	5,100205105	4,425
16	0,462	4,566077196	6,272755398	10,6501328	4,588058194	6,624934629	5,481468795	4,763
17	0,495	4,919439938	6,703528742	11,68843967	5,00981054	7,215740261	5,873351624	5,082
18	0,528	5,267429448	7,163502192	12,78841263	5,461927387	7,903729865	6,283843286	5,382
19	0,561	5,570356636	7,629477234	13,94870602	5,815024209	8,63011697	6,643446367	5,676
20	0,594	5,80779689	8,134563816	15,08818778	6,206112829	9,399158759	7,045882493	6,035
21	0,627	6,055795755	8,678496593	16,24581773	6,547470114	10,17977257	7,4744327	6,390
22	0,66	6,37880983	9,26087019	17,42309556	6,822020101	10,99886098	7,937936049	6,738
23	0,693	6,660967315	9,788877614	18,52471306	7,147855432	11,8297804	8,422138196	7,098
24	0,726	7,017850546	10,38580921	19,74687562	7,476905524	12,66147328	8,934702678	7,512
25	0,759	7,345536493	11,03392893	21,00784292	7,78728221	13,47645427	9,418716461	7,894
26	0,792	7,624468151	11,71663269	22,24751252	8,064465729	14,3568269	9,888562771	8,255
27	0,825	7,903714939	12,41822614	23,43156142	8,277424859	15,32329787	10,30654166	8,601
28	0,858	8,197189283	13,10474539	24,54026547	8,413704862	16,35429489	10,72432633	8,925
29	0,891	8,504553696	13,79673335	25,65367447	8,527760387	17,47761174	11,00359056	9,237
30	0,924	8,811928509	14,48882132	26,76678347	8,637760387	18,60071861	11,28276056	9,549
31	0,957	9,119303322	15,18090929	27,87989247	8,747760387	19,72382648	11,56193056	9,861
32	0,99	9,426678135	15,87299726	28,99300147	8,857760387	20,84693435	11,84110056	10,173
33	1,023	9,734052948	16,56508523	30,10611047	8,967760387	21,97004222	12,12027056	10,485
34	1,056	10,04142776	17,2571732	31,21921947	9,077760387	23,09315009	12,39944056	10,797
35	1,089	10,34880257	17,94926117	32,33232847	9,187760387	24,21625796	12,67861056	11,109
36	1,122	10,65617738	18,64135014	33,44543747	9,297760387	25,33936583	12,95778056	11,421
37	1,155	10,96355219	19,33343911	34,55854647	9,407760387	26,4624737	13,23695056	11,733
38	1,188	11,270927	20,02552808	35,67165547	9,517760387	27,58558157	13,51612056	12,045
39	1,221	11,57830281	20,71761705	36,78476447	9,627760387	28,70868944	13,79529056	12,357
40	1,254	11,88567862	21,40970602	37,89787347	9,737760387	29,83179731	14,07446056	12,669
41	1,287	12,19305343	22,10179502	39,01098247	9,847760387	30,95490518	14,35363056	12,981
42	1,32	12,50042824	22,79388402	40,12409147	9,957760387	32,07801305	14,63280056	13,293
43	1,353	12,80780305	23,48597302	41,23720047	10,067760387	33,20112092	14,91197056	13,605
44	1,386	13,11517786	24,17806202	42,35030947	10,177760387	34,32422879	15,19114056	13,917
45	1,419	13,42255267	24,87015102	43,46341847	10,287760387	35,44733666	15,47031056	14,229
46	1,452	13,72992748	25,56224002	44,57652747	10,397760387	36,57044453	15,74948056	14,541
47	1,485	14,03730229	26,25432902	45,68963647	10,507760387	37,6935524	16,02865056	14,853
48	1,518	14,3446771	26,94641802	46,80274547	10,617760387	38,81666027	16,30782056	15,165
49	1,551	14,65205191	27,63850702	47,91585447	10,727760387	39,93976814	16,58699056	15,477
50	1,584	14,95942672	28,33059602	49,02896347	10,837760387	41,06287601	16,86616056	15,789
51	1,617	15,26680153	29,02268502	50,14207247	10,947760387	42,18598388	17,14533056	16,101
52	1,65	15,57417634	29,71477402	51,25518147	11,057760387	43,30909175	17,42450056	16,413
53	1,683	15,88155115	30,40686302	52,36829047	11,167760387	44,43220062	17,70367056	16,725
54	1,716	16,18892596	31,09895202	53,48139947	11,277760387	45,55530849	17,98284056	17,037
55	1,749	16,49630077	31,79104102	54,59450847	11,387760387	46,67841636	18,26201056	17,349
56	1,782	16,80367558	32,48313002	55,70761747	11,497760387	47,80152423	18,54118056	17,661
57	1,815	17,11105039	33,17521902	56,82072647	11,607760387	48,9246321	18,82035056	17,973
58	1,848	17,4184252	33,86730802	57,93383547	11,717760387	50,04773997	19,09952056	18,285
59	1,881	17,72580001	34,55939702	59,04694447	11,827760387	51,17084784	19,37869056	18,597
60	1,914	18,03317482	35,25148602	60,16005347	11,937760387	52,29395571	19,65786056	18,909
61	1,947	18,34054963	35,94357502	61,27316247	12,047760387	53,41706358	19,93703056	19,221
62	1,98	18,64792444	36,63566402	62,38627147	12,157760387	54,54017145	20,21620056	19,533
63	2,013	18,95529925	37,32775302	63,49938047	12,267760387	55,66327932	20,49537056	19,845
64	2,046	19,26267406	38,01984202	64,61248947	12,377760387	56,78638719	20,77454056	20,157
65	2,079	19,57004887	38,71193102	65,72559847	12,487760387	57,90949506	21,05371056	20,469
66	2,112	19,87742368	39,40402002	66,83870747	12,597760387	59,03260293	21,33288056	20,781
67	2,145	20,18479849	40,09610902	67,95181647	12,707760387	60,1557108	21,61205056	21,093
68	2,178	20,4921733	40,78819802	69,06492547	12,817760387	61,27881867	21,89122056	21,405
69	2,211	20,79954811	41,48028702	70,17803447	12,927760387	62,40192654	22,17039056	21,717
70	2,244	21,10692292	42,17237602	71,29114347	13,037760387	63,52503441	22,44956056	22,029
71	2,277	21,41429773	42,86446502	72,40425247	13,147760387	64,64814228	22,72873056	22,341
72	2,31	21,72167254	43,55655402	73,51736147	13,257760387	65,77125015	23,00790056	22,653
73	2,343	22,02904735	44,24864302	74,63047047	13,367760387	66,89435802	23,28707056	22,965
74	2,376	22,33642216	44,94073202	75,74357947	13,477760387	68,01746589	23,56624056	23,277
75	2,409	22,64379697	45,63282102	76,85668847	13,587760387	69,14057376	23,84541056	23,589
76	2,442	22,95117178	46,32491002	77,96979747	13,697760387	70,26368163	24,12458056	23,901
77	2,475	23,25854659	47,01700902	79,08290647	13,807760387	71,3867895	24,40375056	24,213
78	2,508	23,5659214	47,70909802	80,19601547	13,917760387	72,50989737	24,68292056	24,525
79	2,541	23,87329621	48,40118702	81,30912447	14,027760387	73,63300524	24,96209056	24,837
80	2,574	24,18067102	49,09327602	82,42223347	14,137760387	74,75611311	25,24126056	25,149
81	2,607	24,48804583	49,78536502	83,53534247	14,247760387	75,87922098	25,52043056	25,461
82	2,64	24,79542064	50,47745402	84,64845147	14,357760387	76,00232885	25,79960056	25,773
83	2,673	25,10279545	51,16954302	85,76156047	14,467760387	77,12543672	26,07877056	26,085
84	2,706	25,41017026	51,86163202	86,87466947	14,577760387	78,24854459	26,35794056	26,397
85	2,739	25,71754507	52,55372102	87,98777847	14,687760387	79,37165246	26,63711056	26,709
86	2,772	26,02491988	53,24581002	89,10088747	14,797760387	80,49476033	26,91628056	27,021
87	2,805	26,33229469	53,93789902	90,21399647	14,907760387	81,6178682	27,19545056	27,333
88	2,838	26,6396695	54,63000802	91,32710547	15,017760387	82,74097607	27,47462056	27,645
89	2,871	26,94704431	55,32209702	92,44021447	15,127760387	83,86408394	27,75379056	27,957
90	2,904	27,25441912	56,01418602	93,55332347	15,237760387	84,98719181	28,03296056	28,269
91	2,937	27,56179393	56,70627502	94,66643247	15,347760387	86,11029968	28,31213056	28,581
92	2,97	27,86916874	57,39836402	95,77954147	15,457760387	87,23340755	28,59130056	28,893
93	3,003	28,17654355	58,09045302	96,89265047	15,567760387	88,35651542	28,87047056	29,205
94	3,036	28,48391836	58,78254202	98,00575947	15,677760387	89,47962329	29,14964056	29,517
95	3,069							

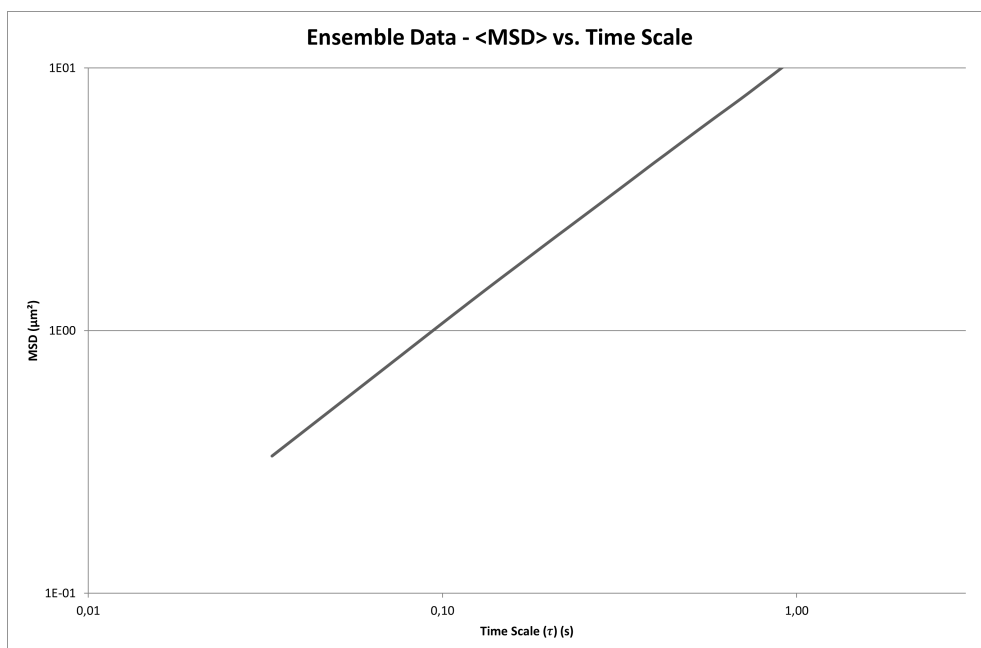


Figure A.2: Individual Particle Analysis spreadsheet (ensemble MSD chart).

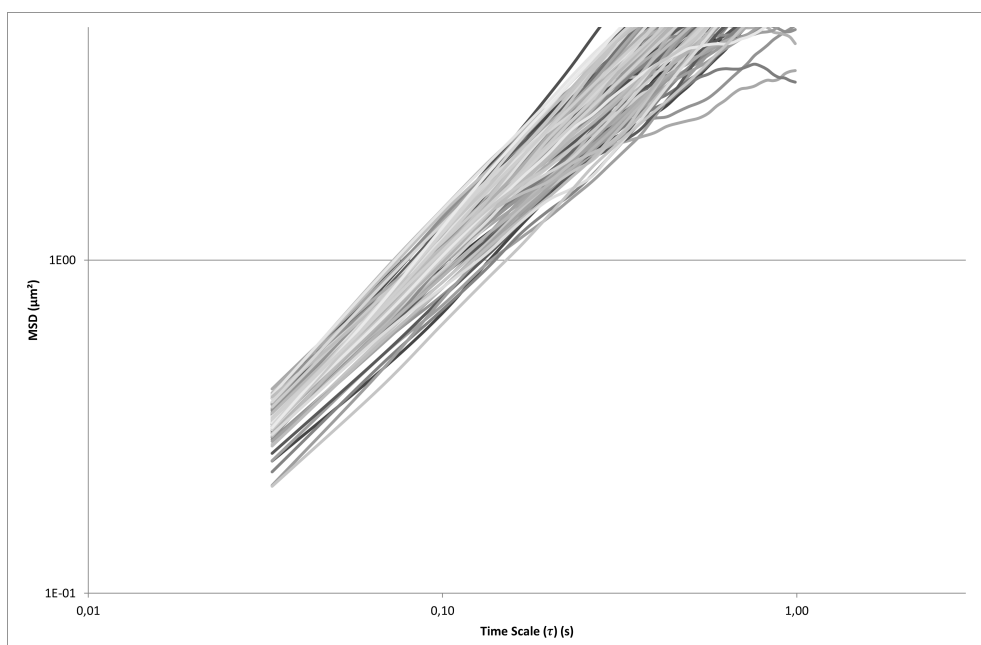


Figure A.3: Individual Particle Analysis spreadsheet (MSD vs Time chart).

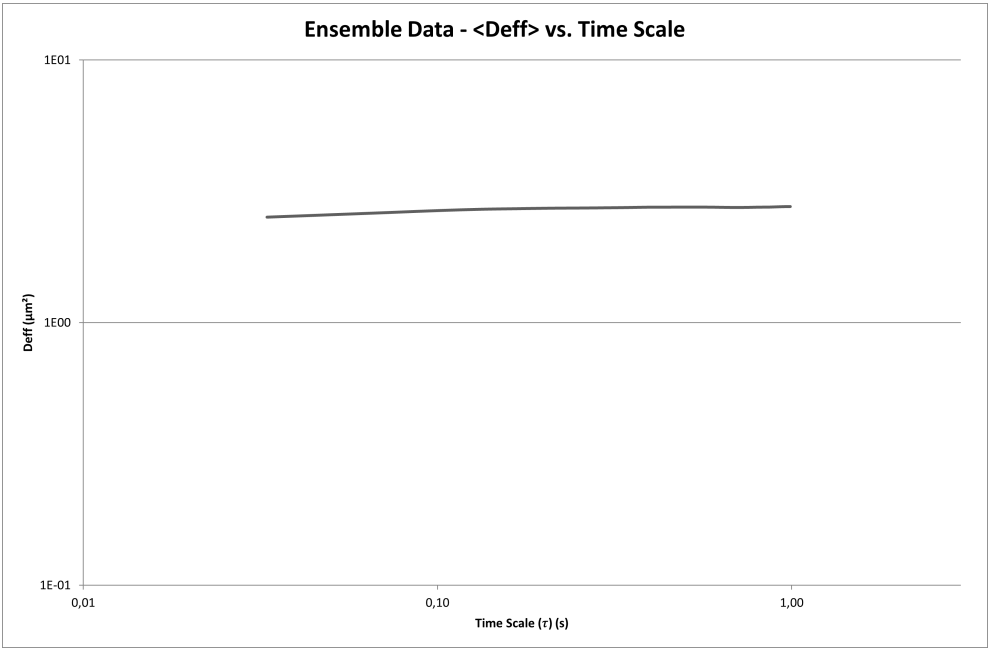


Figure A.4: Individual Particle Analysis spreadsheet ($\langle D_{eff} \rangle$ vs Time editable chart).

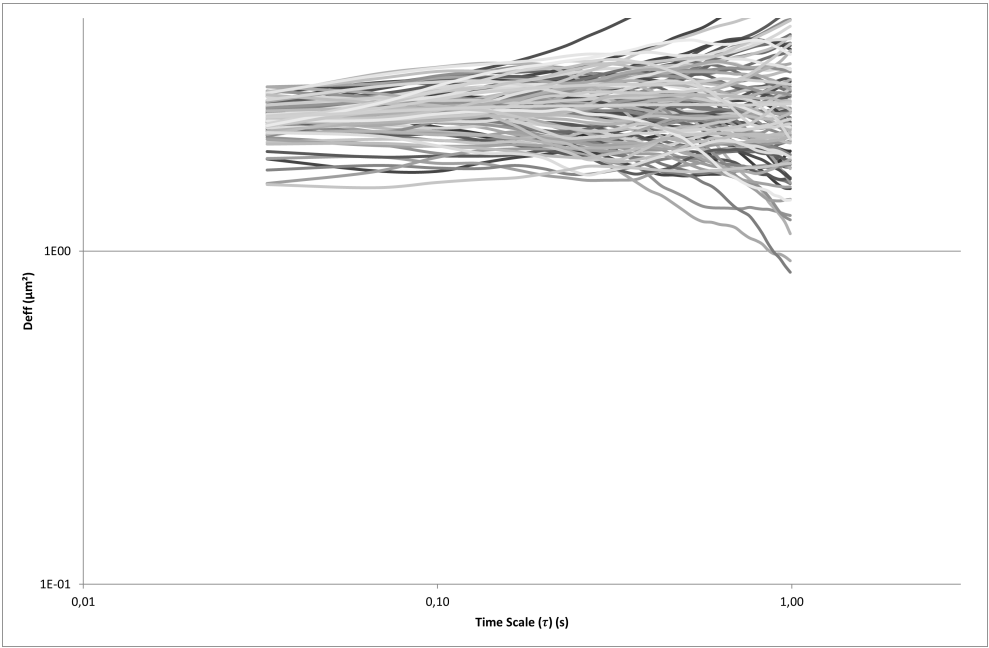


Figure A.5: Individual Particle Analysis spreadsheet (D_{eff} vs Time chart).

	A	B	C	D	E	F	G	
1								
2	Timescale (r) (s)	MSD-LOG 1 (μm^2)	MSD-LOG 2 (μm^2)	MSD-LOG 3 (μm^2)	MSD-LOG 4 (μm^2)	MSD-LOG 5 (μm^2)	MSD-LOG 6 (μm^2)	MSD-I
3	-1,48148606	-0,444281536	-0,474633850	-0,444908085	-0,492581307	-0,476256710	-0,460360994	-0,44
4	-1,180456064	-0,125900346	-0,147772640	-0,094134087	-0,178342705	-0,131297428	-0,157086044	-0,15
5	-1,004364805	0,049321968	0,069687123	0,115557763	-0,035447891	0,049792851	0,037922780	0,03
6	-0,879426069	0,167182893	0,210085232	0,267812607	0,068090390	0,176873780	0,178816905	0,15
7	-0,782516056	0,247211155	0,325495610	0,388232134	0,159944140	0,284153404	0,285510855	0,25
8	-0,70333481	0,312163441	0,410814614	0,491437874	0,240953622	0,369621662	0,375135933	0,33
9	-0,63638802	0,377811135	0,485901460	0,582306381	0,313895178	0,451840573	0,448076559	0,40
10	-0,578396073	0,424675565	0,547386433	0,667509047	0,374251227	0,523842267	0,507163480	0,47
11	-0,527243551	0,471124644	0,603589233	0,741222907	0,429807937	0,583411214	0,559410102	0,51
12	-0,48148606	0,511591967	0,652154983	0,809716397	0,483685124	0,642089694	0,602241473	0,55
13	-0,440093375	0,554010797	0,694779061	0,872399348	0,532631111	0,690601268	0,639882365	0,58
14	-0,402304814	0,590991986	0,731458101	0,930325173	0,580498190	0,734796938	0,675700018	0,61
15	-0,367542708	0,626070206	0,764572932	0,981795211	0,622074703	0,778734324	0,707587642	0,64
16	-0,335358024	0,659543250	0,797458353	1,027355023	0,661628918	0,821181597	0,738896946	0,67
17	-0,305394801	0,691915663	0,826303476	1,067755539	0,699821302	0,858280892	0,768886002	0,70
18	-0,277366077	0,721598727	0,855125398	1,106816641	0,737345922	0,897832088	0,798225346	0,73
19	-0,251037139	0,745883001	0,882494781	1,144533921	0,764551527	0,936016682	0,822393433	0,75
20	-0,226213555	0,764011420	0,910334271	1,178637081	0,792819667	0,973088985	0,847935396	0,78
21	-0,202732459	0,782171219	0,938444497	1,210741576	0,816073525	1,007738075	0,873578236	0,80
22	-0,180456064	0,804739655	0,966651797	1,241125319	0,833912995	1,041347713	0,899707596	0,82
23	-0,159266765	0,823537303	0,990732899	1,267751490	0,854175760	1,072976683	0,925422363	0,85
24	-0,139063379	0,846204115	1,016440340	1,295498391	0,873721893	1,102484243	0,951080105	0,87
25	-0,119758224	0,866023521	1,042730182	1,322381461	0,891385914	1,129575642	0,973991723	0,89
26	-0,101274818	0,882209555	1,068802815	1,347281460	0,906575601	1,157058464	0,995133175	0,91
27	-0,083546051	0,897831268	1,094059564	1,369801230	0,917895247	1,185352244	1,013112963	0,93
28	-0,066512712	0,913664964	1,117428588	1,389879257	0,924987274	1,213631824	1,030370021	0,95
29	-0,050112206	0,930651476	1,139776231	1,409154658	0,930834084	1,243483087	1,048673485	0,96
30	-0,0350112206	0,948651476	1,161776231	1,428154658	0,939834084	1,273483087	1,068673485	0,97

Figure A.6: Transport Mode Characterization spreadsheet (raw analysis data).

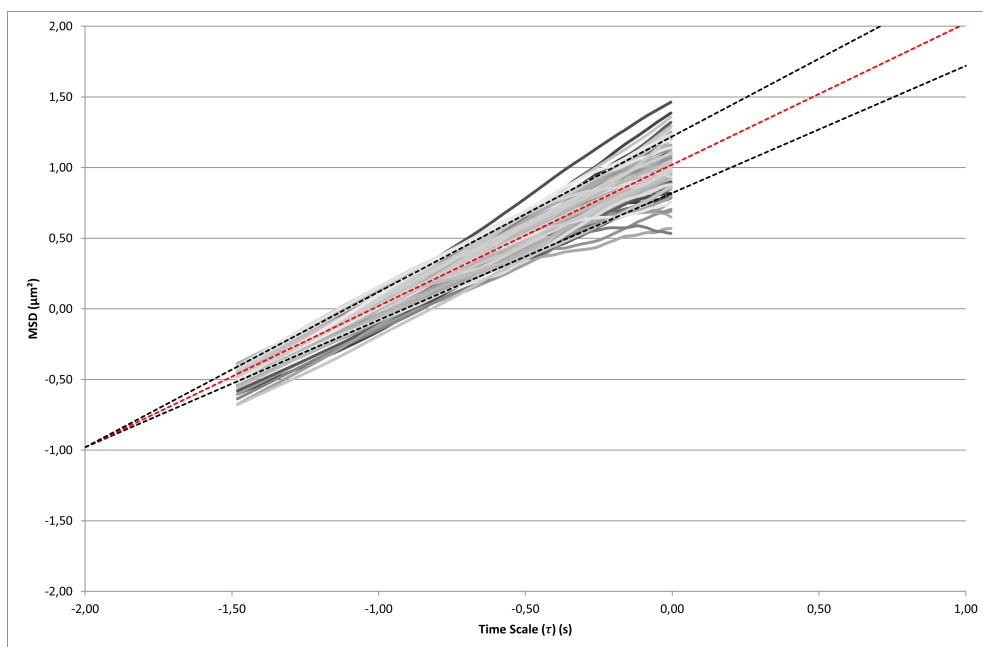


Figure A.7: Transport Mode Characterization spreadsheet (MSD vs Time chart).

	A	B	C	D	E	F
1	Slopes	Slopes (Excel)	R ² (Excel)	Transport Mode	Slope	Count
2	0,946003222	0,946003222	0,999286258	Immobile	0,0-0,199	0
3	1,113317046	1,113317046	0,999057535	Sub-diffusive	0,2-0,899	19
4	1,340696417	1,340696417	0,998223359	Diffusive	0,9-1,199	76
5	1,028013443	1,028013442	0,99645975	Active	1,2+	7
6	1,225965352	1,225965352	0,994306885			
7	1,044505004	1,044505004	0,999415628			
8	0,990424520	0,99042452	0,998715094	<slope> =	0,997907128	
9	0,936409573	0,936409573	0,996765191	N =	102	
10	0,926102028	0,926102028	0,992990995	STD =	0,134222422	
11	0,820440377	0,820440377	0,979635679			
12	1,014209825	1,014209824	0,993315373			
13	0,802135139	0,802135139	0,989021957			
14	0,978573239	0,978573239	0,997081521			
15	1,040239918	1,040239918	0,992301314			
16	1,083384612	1,083384612	0,998257775			
17	0,946599831	0,946599831	0,996841926			
18	1,132902333	1,132902333	0,997483036			
19	1,061521906	1,061521906	0,991163249			
20	0,880342849	0,88034285	0,929800207			
21	1,255023774	1,255023774	0,99123038			
22	1,124852122	1,124852122	0,994266720			

Figure A.8: Transport Mode Characterization spreadsheet (characterization).

	A	B	C	D	E	F	G	H	I	J
1	$D = \frac{k_B T}{6\pi \eta r}$						Timestamp	D ₀	D ₀	D _w / D ₀
2							(s)	(μm ² ·s ⁻¹)	(m ² ·s ⁻¹)	
3							0,99000	2,767226340	2,76723E-12	0,7882
4										
5	D _w (m ² s ⁻¹)	2,18110E-12	=>	D _w (μm ² s ⁻¹)	2,1811					
6	K _B (m ² kg s ⁻²)	13,80649E-24								
7	T (K)	298,15	<=	T (°C)	25					
8	Pi	3,1415927								
9	H ₂ O viscosity (Pa.s)	0,0008900								
10	Radius (m)	0,0000001	<=	Diameter (nm)	225					
11	NOTE: Pa.s = kg m ⁻¹ s ⁻¹									
12										
13	Input cells	These cells are used to input known values								
14	Info cells	Informative cells to help understand the formula								
15	Intermediate cells	These are cells used for the calculation. Must not be changed!								
16	Output cells	Desired results are in these cells.								
17										
18										
19										
20										

Microviscosity calculation

H2O viscosity

Figure A.9: Stokes-Einstein Calculations (D0_Dw) spreadsheet (microviscosity calculation).

	A	B	C
1	Dimensionless viscosity of liquid water at 0.1 MPa		
2	$\bar{\mu} = \sum_{i=1}^4 a_i (\tilde{T})^{b_i}$		
3			
4			
5	i	a _i	b _i
6	1	280,68	-1,9
7	2	511,45	-7,7
8	3	61,131	-19,6
9	4	0,45903	-40
10	https://doi.org/10.1063/1.3088050		

Figure A.10: Stokes-Einstein Calculations (D0_Dw) spreadsheet (H2O viscosity [97]). This is an accessory tab to help on the value of H2O viscosity (cell B9).

Appendix B

Differences in NP transport of 200 nm COOH-PS nanoparticles by individual analysis of MSD and D_{eff}

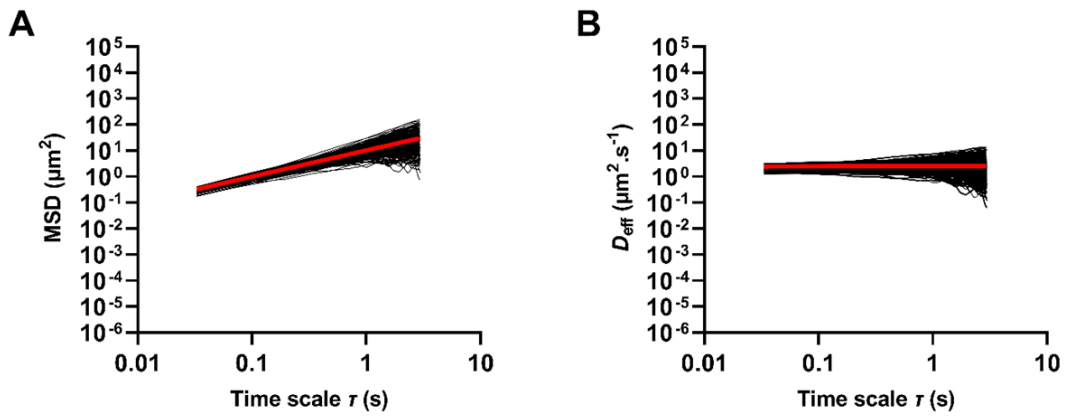


Figure B.1: Individual (A) MSD and (B) D_{eff} of 200 nm COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in water as a function of time scale. Ensemble values are also presented in red in both graphs.

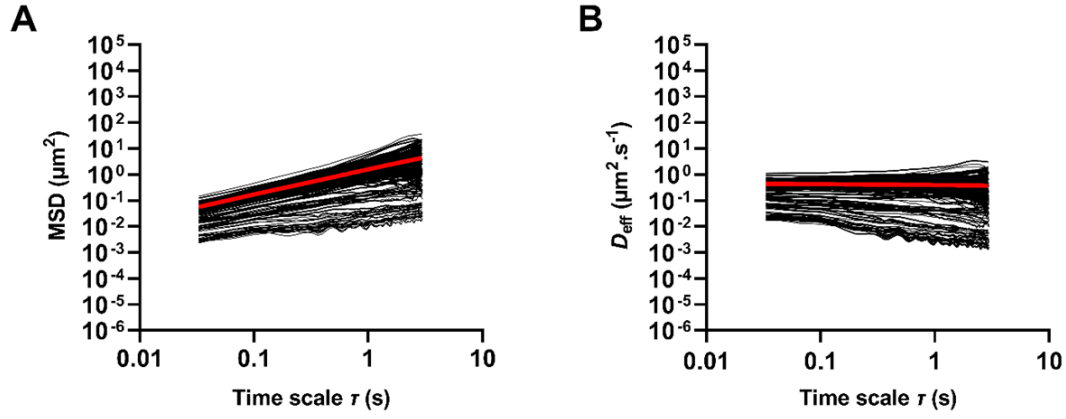


Figure B.2: Individual **(A)** MSD and **(B)** D_{eff} of 200 nm COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in mucus surrogate containing 3% mucin as a function of time scale. Ensemble values are also presented in red in both graphs.

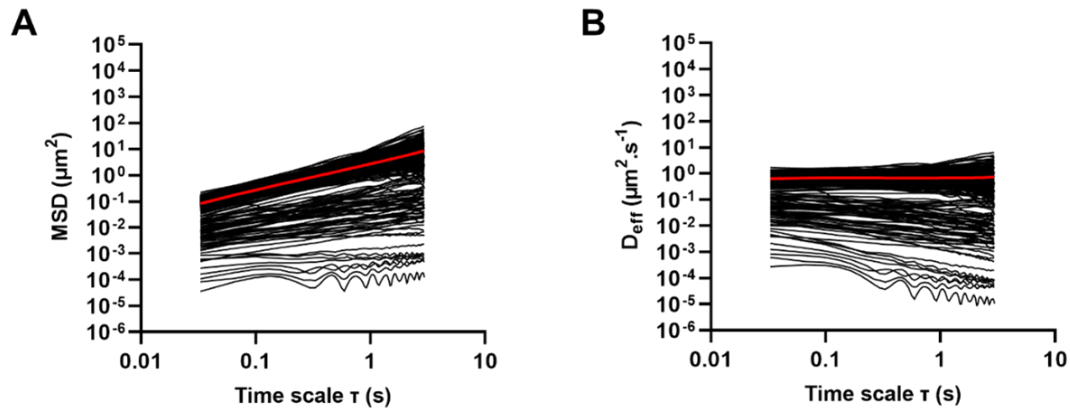


Figure B.3: Individual **(A)** MSD and **(B)** D_{eff} of 100 nm COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in mucus surrogate containing 3% mucin as a function of time scale. Ensemble values are also presented in red in both graphs.

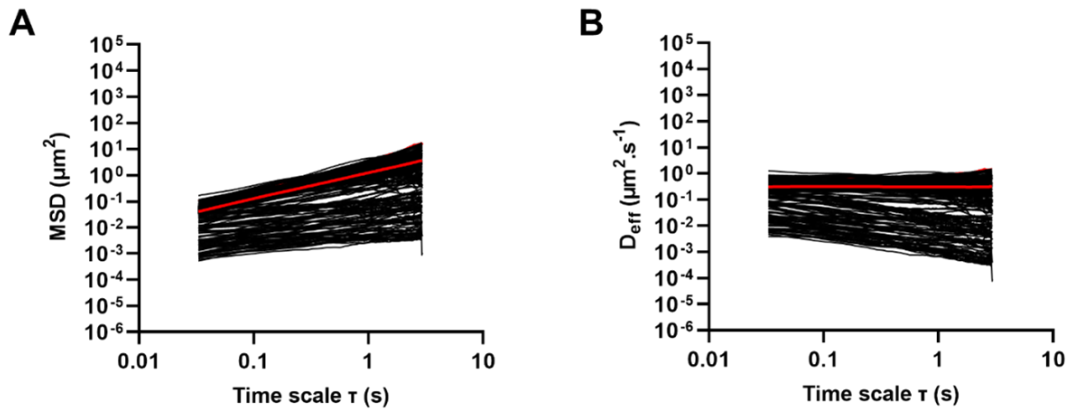


Figure B.4: Individual (A) MSD and (B) D_{eff} of 100 nm COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in mucus surrogate containing 5% mucin as a function of time scale. Ensemble values are also presented in red in both graphs.

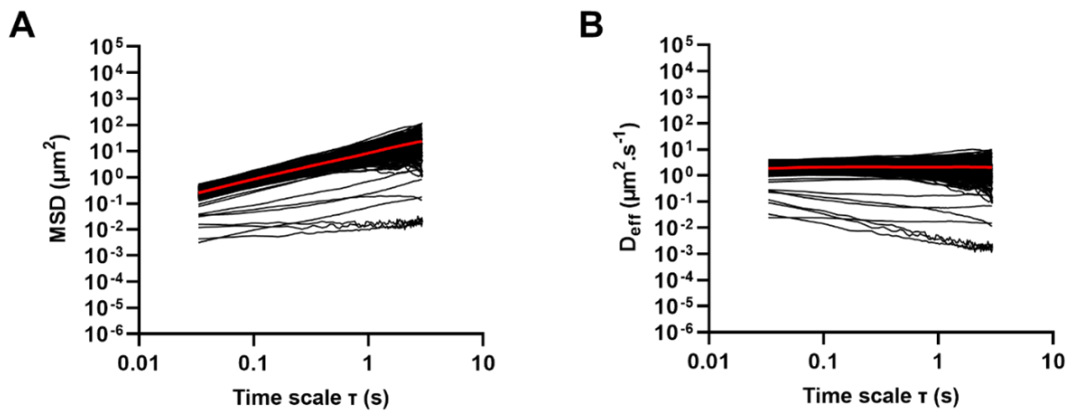


Figure B.5: Individual (A) MSD and (B) D_{eff} of 100 nm PEG-modified COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in mucus surrogate containing 3% mucin as a function of time scale. Ensemble values are also presented in red in both graphs.

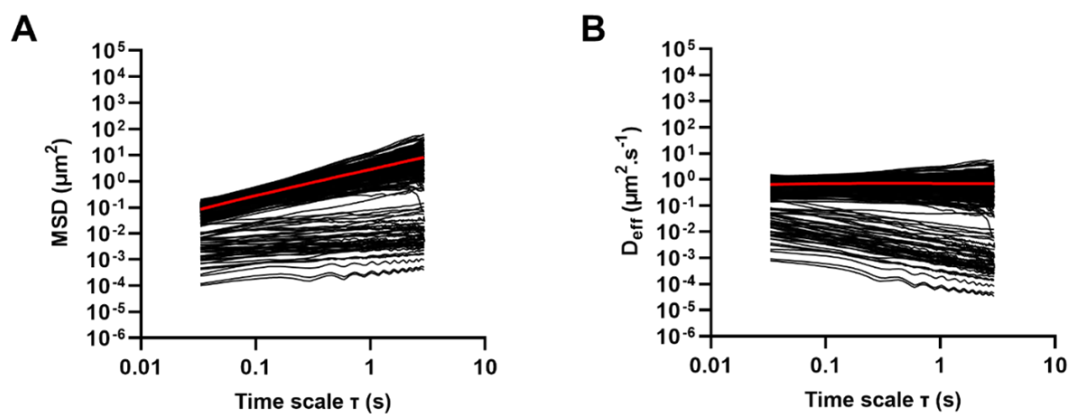


Figure B.6: Individual **(A)** MSD and **(B)** D_{eff} of 100 nm PEG-modified COOH-PS nanoparticles (≥ 100 tracked particles per experiment; $n = 3$) in mucus surrogate containing 5% mucin as a function of time scale. Ensemble values are also presented in red in both graphs.

Appendix C

Experimental protocol

C.1 Materials

- 1.5×1.6 cm gene frames ($65 \mu\text{l}$) (see Figure [C.1](#))
- Red fluorescent carboxylate-modified polystyrene (COOH-PS) nanoparticles with nominal mean diameter of 100 nm or 200 nm
- Phosphate buffered saline (PBS)
- Purified mucin
- Amicon Ultra-0.5 mL (100 kDa MWCO) centrifugal filters
- Poloxamer 407
- Spatula
- Weigh boat
- Scale
- Micropipette/tips
- Fluorescence microscope

C.2 Preparation of surrogate mucin samples

- Reconstitute mucin in isotonic PBS (pH 7.4) at $30 \text{ mg} \cdot \text{mL}^{-1}$ or $50 \text{ mg} \cdot \text{mL}^{-1}$ with the aid of a vortex mixer
- Allow the mucus simulant to equilibrate for 30 minutes at room temperature

C.3 Preparation of nanoparticle solutions

- Wash fluorescent COOH-PS nanoparticles once with ultrapure water using centrifugal filters (4,300 rpm, 10 min) to remove traces of sodium azide
- Resuspend nanoparticles in the same solvent at concentrations varying between 5 and 20 $\text{mg} \cdot \text{mL}^{-1}$
- Store dispersions at 4 °C until further use
- Incubate 100 nm nanoparticles in a 1% (wt/vol) polymer solution overnight to achieve coating with poloxamer 407
- Wash nanoparticles three times again before usage, as detailed above
- Determine the hydrodynamic diameter, polydispersity index (Pdl) and zeta potential by dynamic light scattering and laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK)
- Dilute nanoparticles between 0.1 and 0.01 $\text{mg} \cdot \text{mL}^{-1}$ in 10 mM sodium chloride (pH 5.8) at 25 °C:
 - Prepare in 2 ml eppendorf tubes:
 - * Dilution 1 (D1): 990 μl PBS + 10 μl nanoparticle solution
 - * Dilution 2 (D2):
 - 875 μl PBS + 125 μl D1 (for 200 nm nanoparticles); or
 - 985 μl PBS + 15 μl D1 (for 100 nm nanoparticles)

C.4 Experimental protocol

- Pipette 65 μl mucus simulant into 4 slides. **NOTE:** The number of slides was set to 4 as a suggestion, but it changes according to the research needs.
- Pipette 65 μl aliquots of distilled H₂O into 4 slides. **NOTE:** The number of slides was set to 4 as a suggestion, but it changes according to the research needs.
- Pipette 2 μl of nanoparticle solution D2 on top of the mucus surrogate. **NOTE:** Be sure NOT to inject the pipette tip into the mucus sample - avoid any unnecessary perturbation of mucin structure
- Multiple particle and mucus types can be prepared to be taken to the microscope room, in several slides. Be sure to identify which slide contain which types of media and nanoparticles
- Seal the slides with a coverslip (make sure it is not exposed to light)
- Let the samples sit at room temperature for 1 hour prior to video microscopy - this allows the particles to disperse and the sample to equilibrate

C.5 Video acquisition

- After 1 hour, bring all the gene frames to the microscope and fix them, one at a time, with the slide holder
- Turn on the Leica DMI6000 inverted epifluorescence microscope
- Adjust the microscope filter to TX2
- Open LAS configuration as follows (see Figure C.2):
 - Acquisition tab: x, y, t
 - Enable auto save
 - Video resolution: 512×512
 - Binning: 2×2
 - Light intensity: max
 - Exposure time: 20 ms (for 100 nm nanoparticles); or 5 ms (for 200 nm nanoparticles)
 - Time interval: 33 ms / frame = 30 FPS (Frames per second)
 - Duration: 20 s
- Adjust the sample holder so the gene frame is directly below the fluorescent light
- Click the 'Live' button (Figure C.3) on the left bottom side panel to start streaming the video (Figure C.4)
- Bring the nanoparticles into focus using the controls on the video window (Figure C.5)
- Create Project for each gene frame, to maintain the work organized (Figure C.6)
- In each project, capture at least 6 videos with approximately 50 nanoparticles in the field of view, as described below:
 - Click Start button to start recording (Figure C.7)
 - Once the recording concludes, save the video file in .TIFF format (Figure C.8)

C.6 Video analysis

- Particle tracking videos were analyzed using an ImageJ/Fiji plugin titled 'MosaicSuite'
- Upload a video file by selecting the 'Open' option from 'File' menu. On the dialog window, browse to the folder containing the videos and select a video file (Figure C.9)
- Once the video has loaded, convert it to grayscale 8-bit .tif time-lapses by selecting 'Image' → 'Type' → '8-bit' (Figure C.10)

- Select 'Process' → 'Subtract Background...', set 'Rolling ball radius' to 10 pixels and click the 'OK' button (Figure C.11A)
- Select 'Process' → 'Filters' → 'Median...', set 'Radius' to 1 pixel and click the 'OK' button (Figure C.11B)
- Run MosaicSuite plugin by selecting 'Plugins' → 'Mosaic' → 'Particle Tracker 2D/3D' (Figure C.12)
- Once selected, the plugin window 'Particle Tracker...' opens. Select the following configurations for optimal detection (Figure C.13)
 - Particle detection:
 - * Radius: 3
 - * Cutoff[0-1]: 0.01
 - * Per/Abs: 0.100
 - Particle Linking:
 - * Link range: 2
 - * Displacement: 10.00
 - * Dynamics: Brownian
- The 'Preview Detected' button can be used to view the detected particles with current parameters, which can help optimizing the parameters
- Other adjusts
- When the parameters are satisfactory, click the 'Ok' button to start analysis
- Once the analysis is concluded, the 'Results' window will show. Click the 'All Trajectories to Table' button (Figure C.14)
- On the new 'Results' window showing the analysis raw data, select 'File' → 'Save As' to export the raw data in .csv file format (Figure C.15)
- Once all the .csv files, from all the captured videos, are exported, proceed to the data analysis using MPThub.

C.7 Data analysis (MPThub)

- Open the MPThub program
- Select 'Application configuration' from 'Tools' menu to review the analysis configuration (Figure C.16)
- Select 'Diffusivity ranges' from 'Tools' menu to review the diffusivity ranges configuration, if needed (Figure C.16)
- Select option 'Import files...' from the 'File' menu and browse to the folder containing all the exported csv files (Figure C.17). Select the desired files and click 'Open' (it is possible to select multiple files)

- MPTHub will import all the files and show a summary with trajectories information for each file (Figure C.18)
- If there are less than 100 valid trajectories, import more files.
- If there are more valid trajectories than needed, remove a file from the summary:
 - Select the desired file for removal and select option 'Remove selected' from 'Tools' menu (Figure C.16)
- Once there are the desired number of valid trajectories, select option 'Start analysis' from the 'Tools' menu to start data analysis (Figure C.16)
- After the analysis has finished, select 'Export files...' from 'File' menu (Figure C.17) and select the destination folder to save the generated reports

C.8 Figures

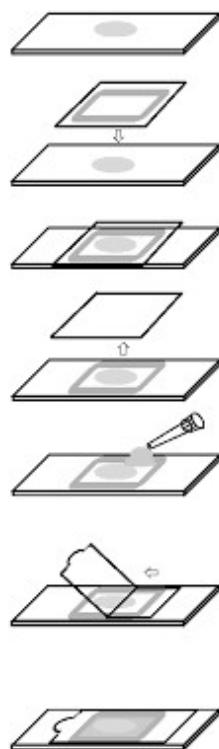


Figure C.1: 1.5×1.6 cm gene frames ($65 \mu\text{l}$) preparation (courtesy of Helena Almeida).

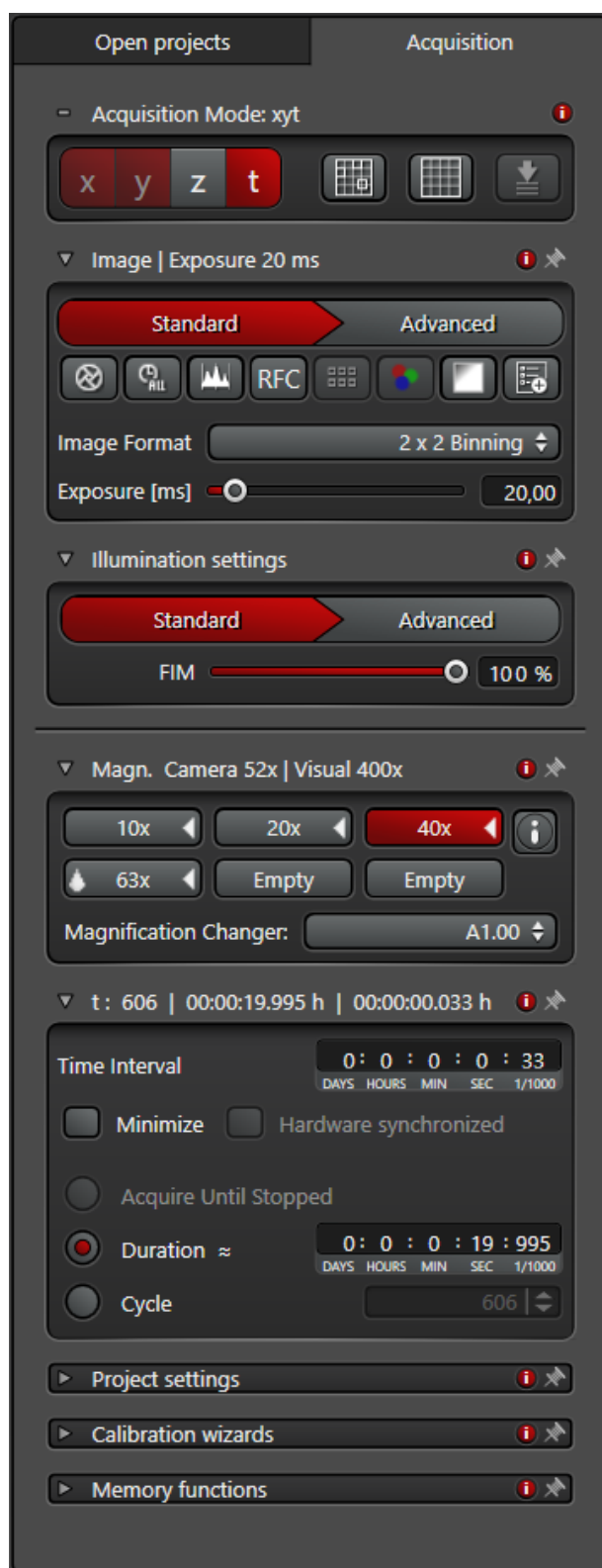


Figure C.2: LAS configuration

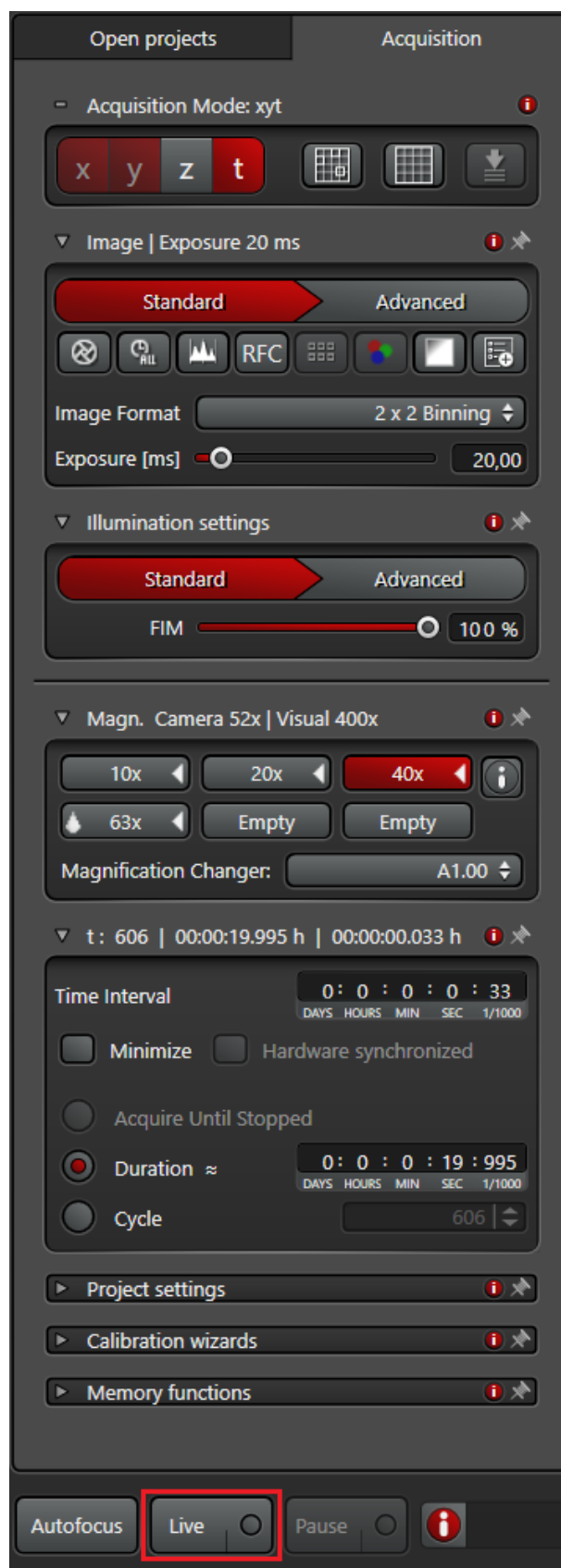


Figure C.3: LAS 'Live' button



Figure C.4: Video live streaming



Figure C.5: LAS video controls

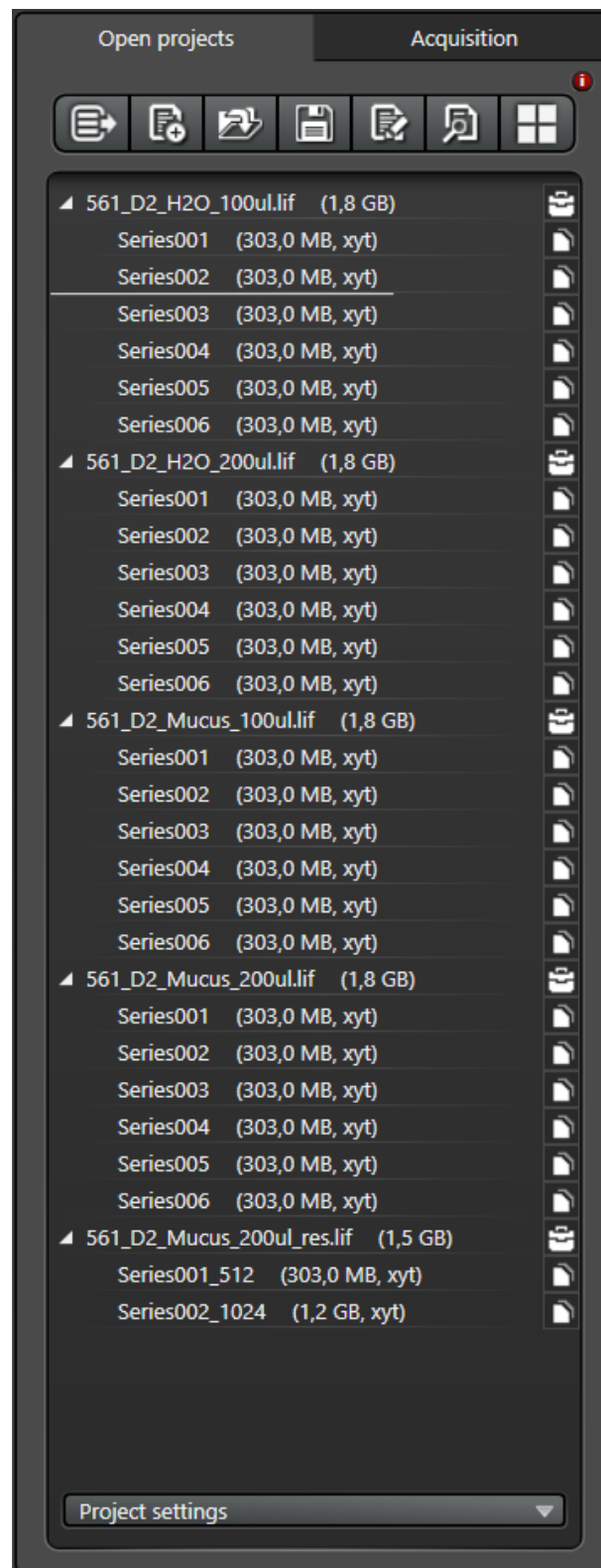
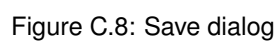
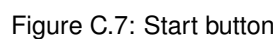


Figure C.6: LAS projects



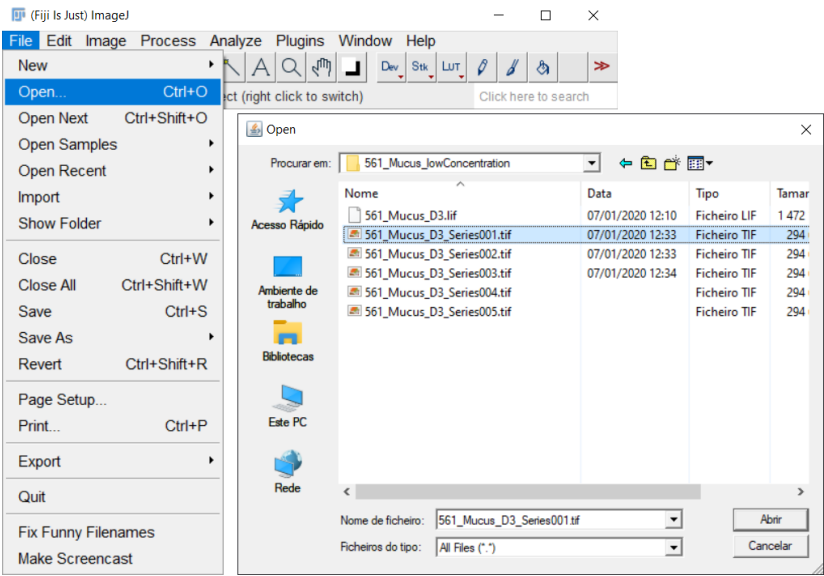


Figure C.9: ImageJ/Fiji open file

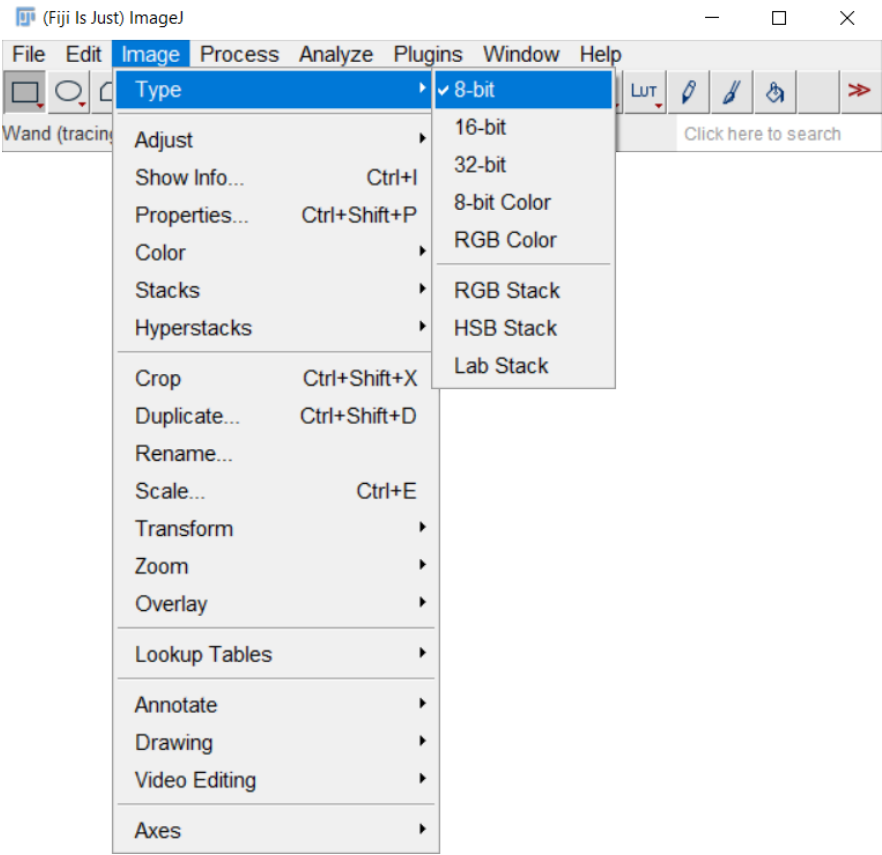


Figure C.10: ImageJ/Fiji grayscale 8-bit setup.

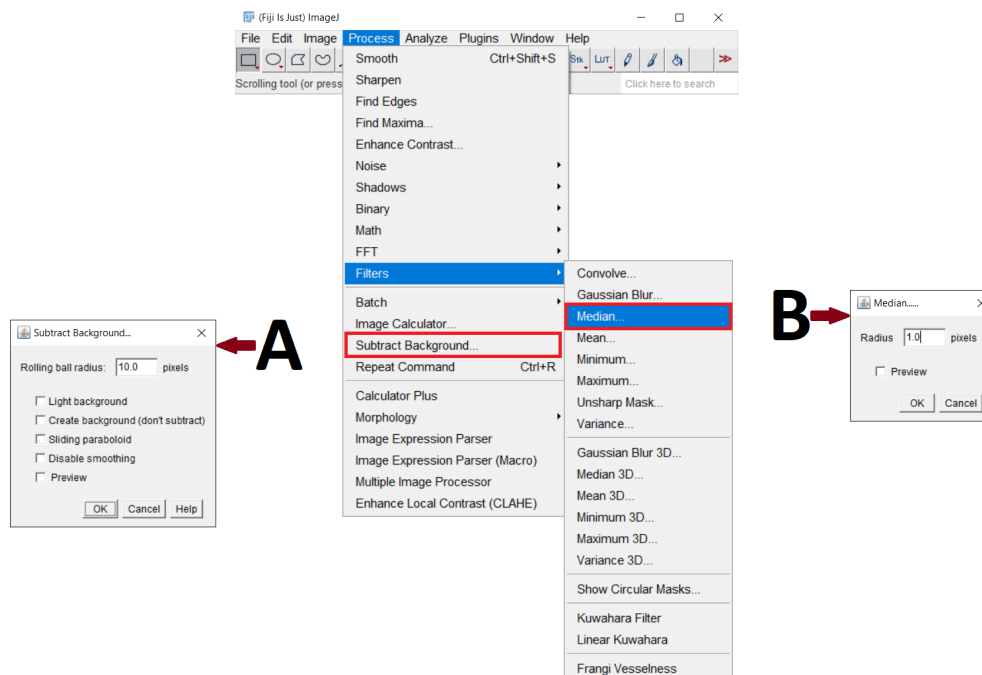


Figure C.11: Process menu. **(A)** Subtract Background option and following setup window; and **(B)** Median filter option and following setup window.

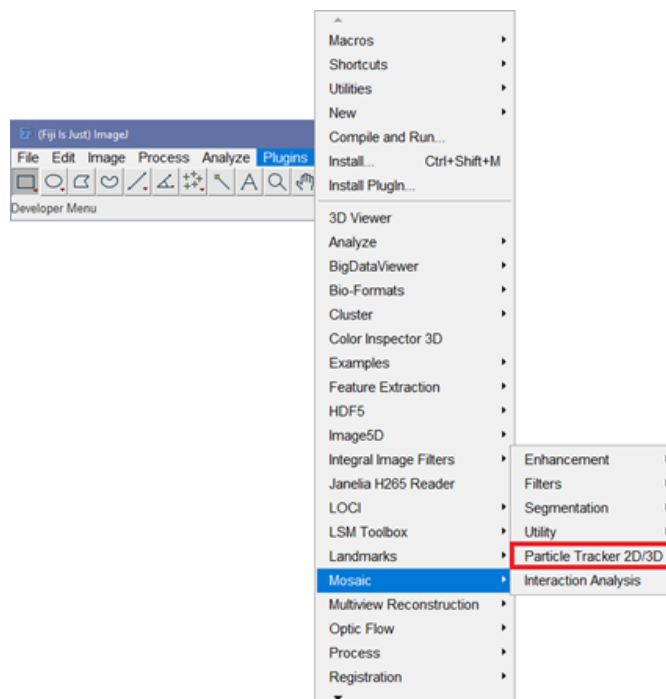


Figure C.12: MosaicSuite plugin menu

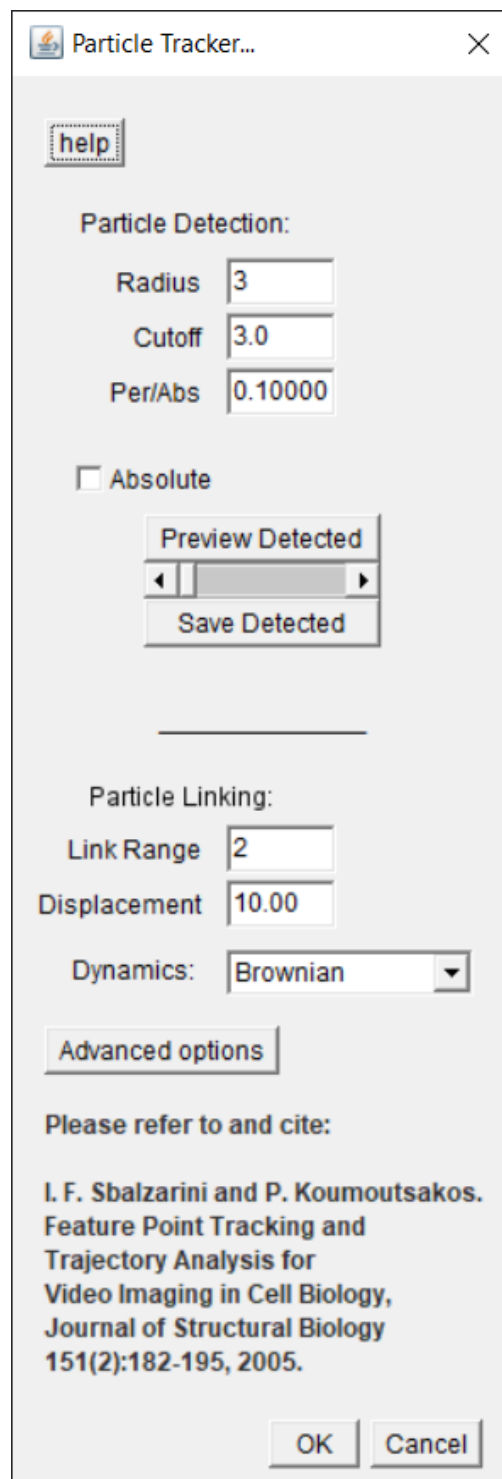


Figure C.13: Particle Tracking optimal configuration

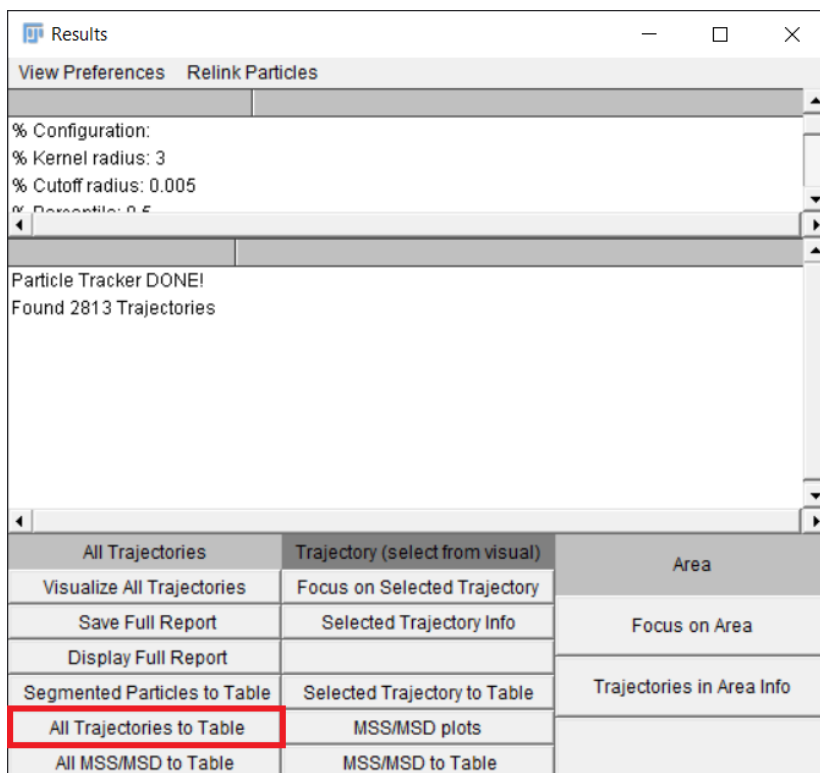


Figure C.14: MosaicSuite 'Results' window

Results

File Edit Font Results

Save As... Ctrl+S

Rename...

Duplicate...

Rename...

Duplicate...

time	x	y	z	m0	m1	m2	m3	m4	NPscore		
6	1	5	99.832	0.796	0	0.169	0.995	1.418	2.231	3.828	42.745
7	1	6	99.637	0.792	0	0.175	0.927	1.242	1.820	2.906	20.294
8	1	7	99.841	1.800	0	0.552	1.309	2.144	3.860	7.502	78.488
9	1	8	97.558	0.961	0	0.231	1.080	1.620	2.682	4.813	34.147
10	1	9	98.729	0.784	0	0.169	0.905	1.195	1.726	2.722	30.031
11	1	10	97.779	0.770	0	0.035	1.073	1.645	2.821	5.324	61.545
12	1	11	97.030	0.758	0	0.045	1.097	1.781	3.344	7.108	81.036
13	1	12	96.212	0.874	0	0.025	1.340	2.528	5.420	12.756	168.892
14	1	13	92.841	0.777	0	0.037	1.078	1.660	2.886	5.580	54.949
15	1	14	92.918	0.857	0	0.197	1.019	1.472	2.347	4.077	21.345
16	1	15	92.272	2.930	0	0.530	1.439	2.579	5.097	10.866	110.767
17	1	16	92.770	3.690	0	0.374	1.507	2.809	5.772	12.779	176.058
18	1	17	92.652	2.674	0	0.450	1.437	2.561	5.022	10.593	142.232
19	1	18	93.284	4.327	0	0.680	1.378	2.367	4.469	9.078	85.484
20	1	19	94.042	5.760	0	0.792	1.383	2.403	4.616	9.593	64.060
21	1	20	95.355	3.891	0	0.697	1.397	2.440	4.696	9.752	75.747
22	1	21	95.816	3.715	0	0.782	1.338	2.245	4.138	8.213	51.431

Figure C.15: MosaicSuite 'Results' table data window

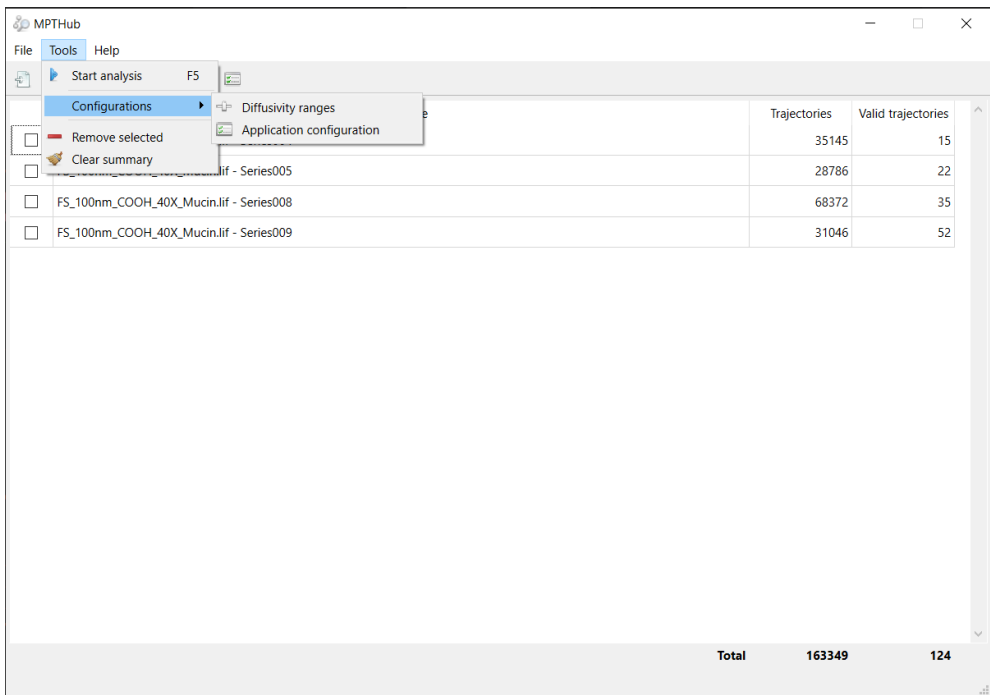


Figure C.16: MPThub 'Tools' menu

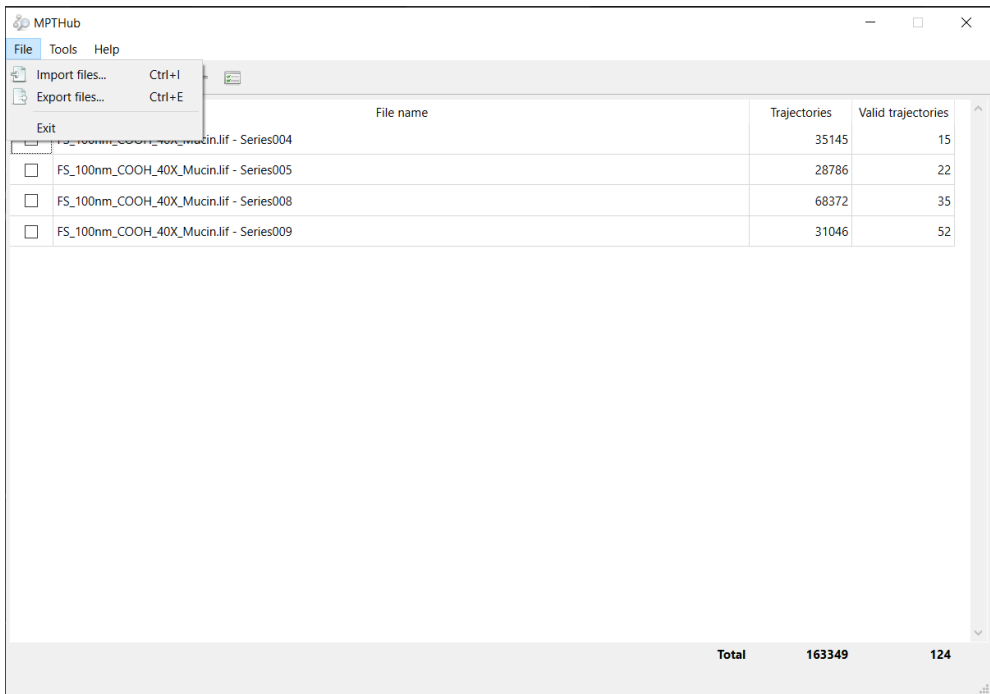
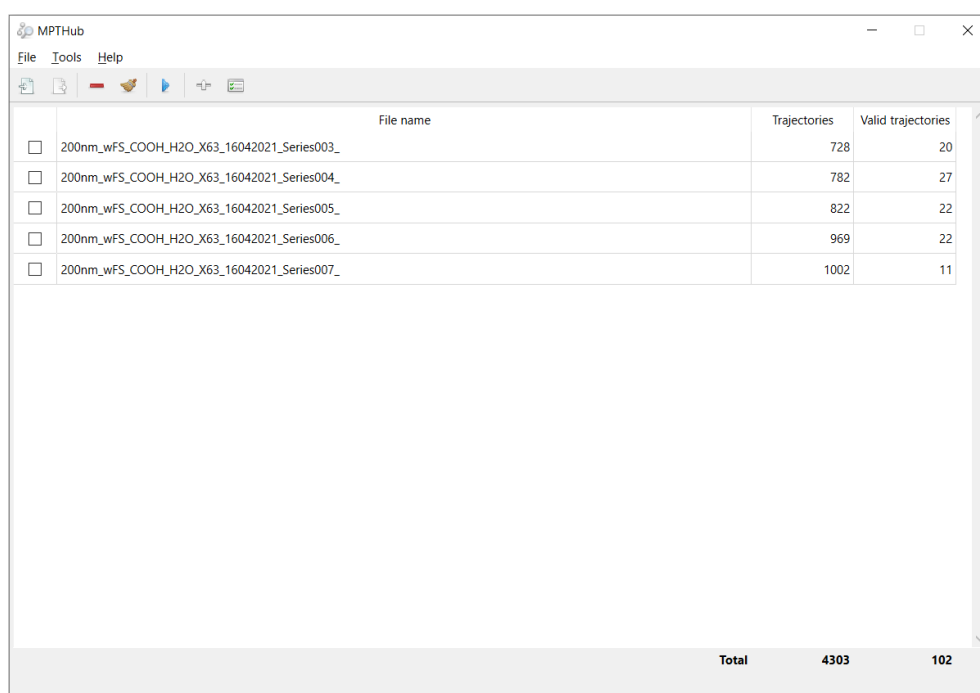


Figure C.17: MPThub 'File' menu



The screenshot shows the MPThub application window. It has a menu bar with 'File', 'Tools', and 'Help'. Below the menu bar is a toolbar with icons for file operations. The main area contains a table with the following data:

	File name	Trajectories	Valid trajectories
☐	200nm_wFS_COOH_H2O_X63_16042021_Series003_	728	20
☐	200nm_wFS_COOH_H2O_X63_16042021_Series004_	782	27
☐	200nm_wFS_COOH_H2O_X63_16042021_Series005_	822	22
☐	200nm_wFS_COOH_H2O_X63_16042021_Series006_	969	22
☐	200nm_wFS_COOH_H2O_X63_16042021_Series007_	1002	11
Total		4303	102

Figure C.18: MPThub summary

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