OMEGA: a software tool for the management, analysis, and dissemination of intracellular trafficking data that incorporates motion type classification and quality control

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Running Head: Sharing particle tracking and motion analysis procedures and results

Abbreviations: DGT, distance from ground truth; MIAPTE, minimum information about particle tracking experiments; MPT, multiple particle tracking; MSD, mean squared displacement; MSS, moment scaling spectrum; ODC, observed diffusion constant; OMERO, open microscopy environment remote objects; SNR, signal to noise ratio; TS, Trajectory Segmentation.

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Abstract

MOTIVATION: Particle tracking coupled with time-lapse microscopy is critical for understanding the dynamics of intracellular processes of clinical importance. Spurred on by advances in the spatiotemporal resolution of microscopy and automated computational methods, this field is increasingly amenable to multi-dimensional high-throughput data collection schemes (Snijder *et al*, 2012). Typically, complex particle tracking datasets generated by individual laboratories are produced with incompatible methodologies that preclude comparison to each other. There is therefore an unmet need for data management systems that facilitate data standardization, meta-analysis, and structured data dissemination. The integration of analysis, visualization, and quality control capabilities into such systems would eliminate the need for manual transfer of data to diverse downstream analysis tools. At the same time, it would lay the foundation for shared trajectory data, particle tracking, and motion analysis standards.

RESULTS: Here, we present Open Microscopy Environment inteGrated Analysis (OMEGA), a cross-platform data management, analysis, and visualization system, for particle tracking data, with particular emphasis on results from viral and vesicular trafficking experiments. OMEGA provides easy to use graphical interfaces to implement integrated particle tracking and motion analysis workflows while keeping track of error propagation and data provenance. Specifically, OMEGA: 1) imports image data and metadata from data management tools such as Open Microscopy Environment Remote Objects (OMERO; Allan et al., 2012); 2) tracks intracellular particles moving across time series of image planes; 3) facilitates parameter optimization and trajectory results inspection and validation; 4) performs downstream trajectory analysis and motion type classification; 5) estimates the uncertainty associated with motion analysis; and, 6) facilitates storage and dissemination of analysis results, and analysis definition metadata, on the basis of our newly proposed Minimum Information About Particle Tracking Experiments (MIAPTE; Rigano & Strambio-De-Castillia, 2016; 2017) guidelines in combination with the OME-XML data model (Goldberg *et al*, 2005).

Availability and implementation: OMEGA is a cross-platform, open-source software developed in Java. Source code and cross-platform binaries are freely available on GitHub at https://github.com/OmegaProject/Omega, under the GNU General Public License v.3.

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Supplementary information: Supplementary Material is available at BioRxiv.org

1 – Introduction

1.1 Description of the problem

Dynamic intracellular processes, such as viral and bacterial infection (Chenouard *et al*, 2009c; Brandenburg & Zhuang, 2007; Sun *et al*, 2013; Mercer *et al*, 2010; Li *et al*, 2017; Pereira *et al*, 2014; Ewers *et al*, 2005), vesicular trafficking (Siebrasse *et al*, 2016; Aoyama *et al*, 2017; Gramlich & Klyachko, 2017; Jandt *et al*, 2011; Jandt & Zeng, 2012; Loerke *et al*, 2009), membrane receptors dynamics (Jaqaman *et al*, 2016; Sergé *et al*, 2008; Block *et al*, 2016; Saxton & Jacobson, 1997), cytoskeletal rearrangement (Applegate *et al*, 2011; Akhmanova & Steinmetz, 2008), focal adhesion reorganization (Berginski *et al*, 2011), gene transcription (Sinha *et al*, 2010), and genome maintenance (Agarwal *et al*, 2011) are important for many clinically relevant fields of study including immune regulation, metabolic disorders, infectious diseases, and cancer. In all of these cases, diverse individual sub-resolution 'particles' (e.g. single molecules, microtubule tips, viruses, vesicles and organelles) dynamically interact with a large number of cellular structures that influence trajectory and speed. The path followed by individual particles varies significantly depending on molecular composition, cargo, and destination. Given the changing and multi-step nature of several of these processes, many questions would benefit from studying them in living cells. The fundamental spatial and temporal heterogeneity of these trafficking processes emphasizes the importance of utilizing single-particle measurements, rather than ensemble averages or flow measurements, in order to gain insight into molecular mechanisms, predict outcome, and rationally design effective therapeutic interventions. The time-resolved visualization of individual heterogeneous intracellular particles by fluorescence-microscopy, coupled with feature point tracking techniques - referred to as Single-Particle Tracking (SPT) (De Brabander *et al*, 1985) or Multiple-Particle Tracking (MPT) (Genovesio *et al*, 2006) - and mathematical analysis of motion, is ideally suited to follow the fate of particles as they progress within the cell, to map fleeting interactions with other cellular components, and to dissect individual transport steps. For example, single viral imaging experiments coupled with MPT have improved understanding of the early phases of viral entry and revealed previously un-recognized entry stages (Brandenburg & Zhuang, 2007; Flatt & Greber, 2017; Sun *et al*, 2013; Greber & Way, 2006; Wang *et al*, 2017; Ewers *et al*, 2005; Helmuth *et al*, 2007; Yamauchi *et al*, 2011).

As a consequence of the steady improvement of the spatiotemporal resolution of microscopic techniques, advances in automated particle tracking and motion analysis (Sbalzarini & Koumoutsakos, 2005; Arhel *et al*, 2006; Jaqaman *et al*, 2008; Chenouard *et al*, 2014; Smith *et al*, 2015; Genovesio *et al*, 2006), and the availability of software tools (Carpenter *et al*, 2012; Schindelin *et al*, 2012; de Chaumont *et al*, 2012; Eliceiri *et al*, 2012; Perry *et al*, 2012; Swedlow & Eliceiri, 2009; Tinevez *et al*, 2016; Jaqaman *et al*, 2008; Kalaidzidis, 2009; Incardona & Sbalzarini, 2014), MPT holds the promise of becoming amenable to multi-dimensional high-throughput data collection schemas (Damm & Pelkmans, 2006; Snijder *et al*, 2012; Rämö *et al*, 2014; Taute *et al*, 2015).

However, many of the fundamental image data management limitations holding back "[…] the routine application of automated image analysis […] to large volumes of information generated by digital imaging" (verbatim from: Swedlow *et al*, 2003) are still in place even fourteen years after the initial identification of the problem . As a case in point, the utilization of viral particle tracking to draw direct real-time correlations between alterations in viral mobility and underlying perturbations in the viral and cellular states, remains a considerable challenge even at low-throughput, and it is difficult if not impossible to scale to the systems biology level (Arhel *et al*, 2006; McDonald *et al*, 2002; Mamede *et al*, 2017; Mamede & Hope, 2016; Sood *et al*, 2017). As a result, most virology studies to date rely on biochemical and genetic analyses conducted in bulk and on the microscopic analysis of fixed cells, which fail to capture viral heterogeneity and the complexity of viral infection processes. Moreover, when particle tracking data is obtained, the datasets produced at individual laboratories are difficult to compare with analogous data generated at other times or places, making integration with different data types, and meta-analysis, impossible.

This situation reveals the presence of an unmet need for tools that would allow the management of large datasets of intracellular trafficking data in a manner that would speed up the analysis workflow, facilitate results inspection, quality control, and validation, foster dissemination and meta-analysis, and, ultimately, lay the foundation for a particle tracking data community.

1.2 Statement of purpose

A major hurdle preventing particle tracking from becoming a routine high-throughput cell biology technique, the results of which can be reproduced and compared across different data production sites, is related to the size and complexity of the data. The number of particles within each cell may be in the hundreds, images typically contain MBs of data, experiment may produce thousands of images and the correct interpretation of results depends on the knowledge of the experimental, optical and image-analysis context. Hence it follows that automated image acquisition, processing, and analysis have to be closely coupled with robust and standardized data management methods, and with accurate accounting of error propagation and data provenance, in order to tackle the problem. Although software tools exist to execute some steps of the particle tracking workflow, tools for several key data management and error evaluation functions do not exist (Table I) (Tinevez *et al*, 2016; de Chaumont *et al*, 2012).

To fill this void we are developing a cross-platform, open-source software called Open Microscopy Environment inteGrated Analysis (OMEGA; Figure 1), which provides a rich graphical user interface (GUI; Figure 2; Supplemental Information 1) to aid the user with particle tracking data production, analysis, validation, and visualization. OMEGA carries out these functions within the framework of our newly proposed Minimum Information About Particle Tracking Experiments (MIAPTE) guidelines (Rigano & Strambio-De-Castillia, 2016; 2017), so that management, annotation, storage, and dissemination of the entire data cascade, is accomplished in a standardized manner, permitting results reproduction and comparison across laboratories.

While OMEGA is intended to assist experimental biologists wishing to quantitate in real time the movement of intracellular particles (i.e. vesicles, virions and organelles), to analyze image data stored within OMERO (Allan *et al*, 2012), it also provides the basic infrastructure for improved annotation, error monitoring, standardization and dissemination in the particle tracking field, facilitating re-interpretation and meta-analysis. The benefit of this approach has been well established in other fields, where data management and dissemination infrastructure is more mature (Data models to GO-FAIR., 2017; Wilkinson *et al*, 2016; UniProt Consortium, 2015; Benson *et al*, 2012; Berman *et al*, 2003; Wenger *et al*, 2000). The development of user-oriented, freely available, data storage and analysis systems for particle tracking data thus offers a timely next step for the field. By unifying the entire image processing and analysis workflow, and by combining it with standardized data management and error propagation handling, OMEGA extends what is currently available, further reduces the need for users to transfer data manually across several downstream analysis tools, and lays the foundation for a particle tracking data dissemination and meta-analysis ecosystem. All OMEGA algorithmic components were tested on artificial image and trajectory data (as described in Supplemental Information 1). All OMEGA supported motion analysis workflows were validated using standard MPT benchmarking datasets (Chenouard *et al*, 2014) as well as real-life datasets depicting retroviral particle trafficking within living human cells (Clark *et al*, 2013; Pereira *et al*, 2012).

2 – Tool description and functionality

2.1 Multiple particle tracking and motion analysis workflow

In a typical particle tracking experiment (Figure 1A), time series of image-frames are recorded from living cells. If the image quality is sufficient, and the spatiotemporal resolution is adequate, MPT algorithms are then used to convert movies depicting particle motion (i.e., viral particles in the example shown) into statistical ensembles of individual trajectories specifying the coordinates and fluorescence intensity of each particle across time (Saxton, 2008; Rust *et al*, 2011). Subsequently, in a process that is often referred to as motion analysis (reviewed in, Meijering *et al*, 2012; Brandenburg & Zhuang, 2007), trajectories are used as input to compute quantitative measures describing the motion state of individual particles as well as their displacement, velocity, and intensity. The aim of this process is to correlate biochemical composition and functional readouts with particle dynamics and ultimately provide readouts that can be used to interpret the mechanisms governing motion and underlying intracellular interactions.

The OMEGA application (Figure 1; Supplemental Information 1) carries out all steps of the complete tracking and motion analysis workflow described above. In addition, because of its flexibility and modularity, it can also be used to support other data-flows depending on user needs (Supplemental Figure 1). OMEGA operates by way of a rich graphical user interface (GUI; Figure 2; Supplemental Information 1), a modular structure and a relational database in the back end to store data arising from image analysis (Figure 3 and

Supplemental Figure 2). The functional logic of the OMEGA Java application (Supplemental Information 1) is organized around six analysis and data management modules (i.e., Image Browser, Particle Tracking, Signal-to-Noise Ratio (SNR) Estimation, Trajectory Manager, Tracking Measures, and Data Browser; Figure 3 and Supplemental Figure 2, solid lines boxes) which in turn implement nine interchangeable plugins (Figure 3, dashed lines boxes) that work sequentially to implements the typical steps of particle tracking and motion analysis experiments (Figures 1 and 4). At time of writing, workflows supported by OMEGA are mainly interactive, requiring user supervision at each subsequent step. In subsequent releases, we plan to develop batch processing of entire image datasets. Extensive validation of OMEGA components was conducted (Supplemental Information 1). Additional validation results will be presented in a parallel manuscript (Rigano *et al*).

2.2 Data selection and loading

OMEGA supports two data import modalities, which are designed to assist users in the task of preserving data provenance links between different data processing steps. The first modality utilizes an Image Browser plugin (Figure 2-2 and 3) to import of image data and metadata from available repositories. The second modality utilizes the Data Browser plugin (Figure 2-14) to directly import pre-computed particles or trajectories.

2.1.1 Image Browser:

OMEGA is designed to import *BioFormats* (OME Consortium, 2017) compatible image data and metadata from available image repositories. At the time of writing OMEGA ships with a custom-designed OMERO Image Browser (Figure 2-2 and 3), which provides a minimal interface for the user to navigate through the OMERO (Allan *et al*, 2012) Project, Dataset, and Image hierarchy, display available content belonging to a specific user in either a list or grid mode, select images to be analyzed and import them into OMEGA. This interface is specifically designed to visualize relevant image information while at the same time avoiding over-cluttering and undue duplication of functionalities already available elsewhere. Only image thumbnails and metadata essential for image selection are shown, while additional viewing options are delegated to available OMERO clients (Allan *et al*, 2012).

2.1.2 Trajectory import in the Data Browser

In order to promote interoperability, users can use the Data browser module (Figure 2-12) to import particles and trajectories previously computed using third-party applications, into OMEGA for further processing and downstream analysis. If images corresponding to these particles and trajectories are available in OMEGA, the detection and linking results can be associated directly with them and made available in the Data Browser for all subsequent data navigation and selection operations. Alternatively, these externally computed tracking data can be associated with a specifically designed “Orphaned Analyses” element. Examples of analyses that were performed on imported, pre-computed trajectories are presented in Figures 5B and 6 and

Supplemental Figure 3.

2.3 Particle Tracking: detection and linking of spots to form trajectories

Once image time-series have been selected and uploaded into OMEGA, the particle tracking module (Figure 2-5) converts movies depicting particle motion (Figure 2-4) into trajectories (Figure 2-6) consisting of the coordinates and fluorescence intensities of each particle across time (Saxton, 2008; Rust *et al*, 2011). Particle Tracking is generally subdivided into two independent steps: 1) Particle Detection: identifies the centers of individual fluorescent spots that are significantly distinguishable from local background and estimates their sub-pixel coordinates and intensities in each time frame. 2) Particle Linking: generates trajectories by linking the position of each bright spot in one time frame with its position in subsequent frames.

Recent systematic and objective comparisons of available particle tracking algorithms on standardized benchmarking data sets (Chenouard *et al*, 2014; Saxton, 2014), have shown that no single algorithm or set of algorithms can optimally solve all tracking problem sets. What is clear is that for any given experimental context and scientific question, multiple algorithms or combinations thereof should be rigorously evaluated before moving to production. This situation makes parameterization and testing of algorithms an essential component of the process. To facilitate this task the OMEGA MPT module is designed to accommodate three different plugin designs: 1) Particle Detection stand-alone; 2) Particle Linking stand-alone; and 3) integrated Particle Tracking plugin merging both detection and linking functionalities into a single element. This flexible plug-in strategy facilitates the integration of diverse tracking algorithms regardless of their specific implementation style and allows the user to "mix and match" compatible particle detection and linking algorithms originating from different sources with a significant improvement in tracking quality and efficiency. In addition, the architecture of the MPT plugin in OMEGA emphasizes modularity and open APIs in order to facilitate the integration of different third-party detection and linking algorithms to be tested on each experimental case. Last but not least, the Data Browser component of OMEGA (Figure 2-14; see below) allows the user to store not only the results of different MPT runs, but also all associated parameter-settings metadata. This enormously facilitates the systematic comparison of runs and the selection of the best tracking method for a given experimental situation of interest.

As a proof of concept, we split the ImageJ-compatible Java implementation of the *MOSAICsuite* Particle Tracker (Sbalzarini & Koumoutsakos, 2005; Incardona & Sbalzarini, 2014) to generate an independent Particle Detection and Particle Linking plugins for OMEGA, which we thoroughly benchmarked (Supplemental Information 1). This algorithm was selected because its performance was formally assessed in a recent objective comparison, where it was found to be one of the most versatile and computationally fastest among the evaluated algorithms (Chenouard *et al*, 2014). In addition, the algorithm has been specifically designed for images with low SNR where prior knowledge of motion modalities is absent, such as during the exploration and optimization phases of a tracking assay. The integration of additional Particle Detection and Particle Linking

plugins in OMEGA is planned for future releases.

2.4 SNR Estimation: image quality control

The accuracy and precision of particle detection as well as that of all downstream trajectory analysis steps depend very closely on the local SNR observed in the immediate surroundings of each identified particle. The OMEGA SNR Estimation module implements plugins that estimate the SNR associated with each tracked particle allowing the identification of images whose quality does not support reliable particle detection and tracking. At time of writing, OMEGA ships with a single local SNR Estimation plugin consisting of a custom Java implementation of the *MOSAICsuite*'s Local SNR Estimation algorithm originally developed by the MOSAIC group in Matlab (Xiao *et al*, 2016; Gong & Sbalzarini, 2016). This routine, which was benchmarked as described (Supplemental Information 1), uses the particle coordinates obtained from the Particle Detection plugin to extract intensity values from each associated image plane and estimate background, noise and SNR pertaining to the area immediately surrounding each particle. Moreover, the plugin computes aggregate SNR values at both the plane and the image level. Briefly, the algorithm first determines the global background and noise associated with the entire image plane where individual particles are localized. It then takes the particle radius as defined by the user to draw a square area around each particle's centroid and identify the brightest pixel within the particle area (i.e., peak intensity). Finally, it estimates local noise, local background and local SNR. Specifically the latter is calculated using three independent models: two are based on the Bhattacharyya distance (i.e., Bhattacharyya Poisson, and Bhattacharyya Gaussian) and the third is based on the method proposed by Cheezum (Cheezum *et al*, 2001).

After calculating local SNR values, the algorithm returns aggregate SNR values at the trajectory, plane and image level, which are utilized two-fold in OMEGA. The global local SNR average over the entire image is used to estimate the minimum detectable Observed Diffusion Constant (ODC; Supplemental Information 1). The global minimum local SNR each given trajectory is utilized to estimate the confidence associated with both ODC and SMSS estimations and with motion type classification (Rigano *et al*). In addition, image averages, minimum and maximum local SNR values can be utilized by the user to evaluate the overall image quality and general performance of the particle detector as specified by the algorithm developer (see below). For example, in the case of the *MOSAICsuite* Particle Detection algorithm, which is currently implemented in OMEGA, an SNR value lower than a threshold of 2 as calculated according to Cheezum (Cheezum *et al*, 2001), indicates to the user that particle detection and motion analysis will not be reliable and better images should be acquired.

2.5 Trajectory Manager: trajectory curation

This module provides interactive graphical support for the inspection of trajectory data quality, correction of linking errors and subdivision of trajectories in segments of uniform mobility.

2.5.1 Trajectory editing

Linking algorithms generally perform satisfactorily provided certain signal to noise ratios, spatiotemporal sampling and observation times criteria are met (Jaqaman & Danuser, 2009). However, despite steady improvements in particle linking methods (Sbalzarini & Koumoutsakos, 2005; Chenouard *et al*, 2014; 2009a; 2009b; Jaqaman *et al*, 2008; Sergé *et al*, 2008; Tinevez *et al*, 2016; Genovesio *et al*, 2006; Ku *et al*, 2007; Jug *et al*, 2014; Kalaidzidis, 2009) it is often necessary to manually inspect and edit individual links. The OMEGA Trajectory Editing plugin is implemented as part of the Trajectory Manager module and uses our custom Trajectory Browser graphical interface to facilitate splitting and merging of trajectories that upon inspection appear to be faulty (Figure 2-9). The most frequent linking errors are due to the following: 1) excessive particle density; 2) insufficiently temporal sampling (i.e., particles move too fast with respect to time interval employed during acquisition); 3) splitting or merging of trajectories, which might result either from an artifact (i.e., two or more particles are close enough that their distance is below the diffraction limit) or from the actual interaction between particles; and 4) particle blinking or moving temporarily out of focus.

2.5.2 Trajectory segmentation

The movement of intracellular objects, such as viral particles or vesicles, is often characterized by frequent switches between different dynamic behaviors the analysis of which can be used to infer interactions between the moving object and its immediate surroundings (Helmuth *et al*, 2007). For example the interaction of viral particles with motor proteins might result in directed motion along microtubules (Brandenburg & Zhuang, 2007; Arhel *et al*, 2006; McDonald *et al*, 2002; Sun *et al*, 2013; Fernandez *et al*, 2015). On the opposite side of the spectrum, interaction with relatively immobile cellular structures such as nuclear pore complexes or membrane rafts, might result in the transient confinement of particles to restricted zones (Schelhaas *et al*, 2008; Burckhardt & Greber, 2009; Kusumi *et al*, 2014; 1993).

Because motion characteristics can be reliably estimated only when trajectories describe stationary and ergodic processes, it is often necessary to decompose trajectories into individual uniform polyline segments to be individually subjected to motion analysis. One additional advantage of this process, herein referred to as Trajectory Segmentation is that events can be defined as specific series of segment types whose frequency can chance as a result of specific molecular or cellular events.

The Trajectory Segmentation plugin of OMEGA provides a specialized version of the Trajectory Browser GUI to assists the user in decomposing trajectories into uniform tracts, each of which can then be analyzed separately (Figure 2-8). The tool allows users to select manually the start and end point of segments and assign a putative motion type to each segment (i.e., *yellow*, confined; fuchsia, sub-diffusive; *blue*, diffusive; *purple*, super-diffusive; *maroon*, directed; Supplemental Table II; Figures 5B and 6; Supplemental Figure 3). This manual method can be used in conjunction with an iterative validation process to reliably obtain homogeneous trajectory segments (see below and Figures 5 and 6). In subsequent releases, we plan to integrate automated

methods for trajectory segmentation in OMEGA (Helmuth *et al*, 2007; Wagner *et al*, 2017; Huet *et al*, 2006; Persson *et al*, 2013; Wang *et al*, 2017).

2.6 Tracking Measures: trajectory analysis, motion type classification and error estimation

Trajectory analysis reduces a sequence of spatial coordinates into scalar quantification parameters that are computed using various averaging techniques applied along the length of the trajectory (Supplemental Table I). The ultimate goal is to gain new understanding about the system under study by computing "biologically meaningful quantitative measures from these coordinates" (verbatim from: Meijering *et al*, 2012). Specifically, OMEGA computes Intensity Tracking Measures (ITM), Mobility Tracking Measures (MTM) and Velocity Tracking Measures (VTM), which are only subject to localization accuracy (i.e., algorithmic systematic bias) and precision (i.e., noise associated random errors) and are therefore considered deterministic (Figures 1, 2 and 4). In addition, OMEGA calculates quantities such as Diffusivity Tracking Measures (DTM) whose values are strongly influenced by sample size, and are therefore statistical (Figures 1, 2 and 4). To facilitate all analysis tasks, the OMEGA Tracking Measure plugins provide a rich interface for users to select trajectory segments using a specialized Segment Browser panel (Figure 2-9), perform quantitative motion analysis on selected segments, examine results in both a tabular and graphical form, export data for downstream processing using third-party application and produce publication grade figures (Figures 4-8). All Tracking Measures plugins (Figure 4) calculate and display both local (i.e., pertaining to an individual particle) and global measures (i.e., pertaining to a whole trajectory or trajectory-segment) as applicable. Furthermore, these plugins can perform limited statistical analysis by computing frequency distributions at the image level. In order to compare results obtained across different images and datasets the user can export results and perform downstream statistical analysis using tools such as R or Matlab (MATLABMathWorks:2018tc; The R Foundation, 2018).

In addition to calculating tracking measures, OMEGA facilitates the classification of either full trajectories or individual uniform segments based on motion type using the ODC vs. SMSS phase space method developed by Ewers et al. (Figures 5 and 6; Supplemental Figure 3; Supplemental Table II; Ewers *et al*, 2005; Schelhaas *et al*, 2008; Sbalzarini & Koumoutsakos, 2005). Last but not least, OMEGA estimates the effect of spot detection uncertainty, limited trajectory length and motion type on the reliability of downstream motion analysis results as an essential pre-requisite for scientists to critically evaluate and compare results (see below).

2.6.1 Trajectory analysis

Intensity Tracking Measures

Fluorescently labeled particles might display changes in fluorescence intensity as a result of addition or loss of labeled components as well as of photo-bleaching and -toxicity. It is therefore often important to report changes in the signal intensity of tracked objects (Figure 4 and Supplemental Table I). For example, when tracking dual-color enveloped viral particles carrying a membrane marker, a sudden drop in intensity might

indicate fusion between the viral envelope and the acidic endocytic compartment (Mamede *et al*, 2017; Sood *et al*, 2017; Padilla-Parra *et al*, 2013; Sood *et al*, 2016; Itano *et al*, 2018). Alternatively, when studying endocytic trafficking, changes in intensity might inform about specific cargo sorting events (Navaroli *et al*, 2012). Most tracking algorithms compute either centroid intensity, peak intensity or both. In addition, the mean intensity of the particle might also be computed when the area of the particle is available. The OMEGA ITM plugin gathers relevant intensity values for each identified particle either directly from the Particle Detection plugin or if necessary from the SNR Estimation plugin and makes them available for visualization on screen as well as for downstream analysis.

Mobility Tracking Measures

Mobility measures are relatively easy to compute and assess the quantity of motion away from the origin or from a reference point, the duration of motion and the persistence along a specific direction (Figures 4 and 6; Supplemental Figure 3; Supplemental Table I). For example, in viral trafficking it is important to quantify what proportion of viral particles move consistently towards the cell center versus those that remain confined near the site of viral entry at the cellular periphery (Yamauchi *et al*, 2011; Navaroli *et al*, 2012; Jaqaman *et al*, 2016; 2011). In OMEGA, local MTM quantify motion associated with a single step (i.e., trajectory link) and include Distance Traveled, Instantaneous Angle and Directional Change. Among global measures computed in OMEGA are: Total Curvilinear Distance Traveled (i.e., the total path length followed by a Brownian particle from start to end of motion; Saxton, 2009), the Total Net Straight-Line Distance Traveled (i.e., the distance between the beginning and the end of motion) and the Confinement Ratio (also known as the meandering index, straightness index or directionality ratio) as a measure of the straightness of trajectories or the confinement of moving particles (Sergé *et al*, 2008; Beltman *et al*, 2009).

Velocity Tracking Measures

Measuring the rate of displacement of moving intracellular objects such as vesicles or viral particles can provide important information about the underlying mechanism of motion (Figures 4 and 6; Supplemental Figure 3; Supplemental Table I). For example, a fast moving viral particle that is also moving in a consisted direction might be moving along microtubules while a particle confined within a membrane raft might remain relatively still for large proportions of time (Gazzola *et al*, 2009; Suomalainen *et al*, 2001; Engelke *et al*, 2011). In OMEGA, VTM include local measures such as Instantaneous Speed. Global measures include Average Curvilinear Speed, Average Straight-line Speed, and Forward Progression Linearity, which gives a measure of how quickly an object is moving away from its origin during the total trajectory time (Meijering *et al*, 2012).

Diffusivity Tracking Measures

Because of their size, intracellular vesicles, virions and all diffraction-limited objects behave like Brownian particles, whose default state is normal diffusion (Saxton, 2009). Under these conditions, alteration in the diffusion state of particles result from molecular interactions that alter the general direction or the rate of motion. As a corollary, the distinction between normal vs. abnormal diffusion represents one of the most

important steps in an attempt to distinguish between phases in which particles are free to move about and phases in which they are engaged in interactions with the surrounding cellular milieu that restrict their mobility. This in turn provides important clues for the understanding of the underlying mechanisms influencing the behavior of intracellular virions, vesicles, and other structures.

All motion processes can be described in terms of the *probability* that a given particle that at time 0 is found at position x(0), moves to position x(t) at time t. Thus, despite the fact that the diffusion coefficient (D; Saxton & Jacobson, 1997) is not constant in time for anomalous diffusion processes, this allows the extension of diffusivity analysis to all types of anomalous diffusion (Ferrari *et al*, 2001; Sbalzarini & Koumoutsakos, 2005). Based on these premises, OMEGA implements a single method to classify the dynamic behavior of individual particles regardless of their motion characteristics and employs the same method for particles whose dynamic behavior changes during the course of motion, as is commonly observed in living systems (Tables I and II).

Specifically, the method implemented in OMEGA (Figures 4-8; Supplemental Figure 3; validated as described in Supplemental Information 1) reproduces well-known methods (Sbalzarini & Koumoutsakos, 2005; Schelhaas *et al*, 2008; Ewers *et al*, 2005), which combines two components: 1) quantitative assessment of the degree to which the motion characteristics of the particle under study deviate from free diffusion; and 2) estimation of the quantity of displacement. In order to assess the diffusivity characteristics of a given particle, the OMEGA DTM plugin (Figures 4-8; Supplemental Figure 3) uses a well-established method based on the observation that the Squared Displacement (SD) of a diffusing particle from the origin of motion grows linearly with time in expectation. After time averages of SD (Landau & Lifshitz, 1960) – Mean Squared Displacement (MSD) – are computed for individual trajectories as a function of calculation time lag (Δt), the scaling behavior (i.e., slope) in plots of log(MSD) *vs.* log(Δt) can be used to calculate D and is sometimes used as an indication of whether the trajectory under study is characterized by normal (slope = 1) or anomalous diffusion (slope $\neq$ 1; Supplemental Table II; Saxton, 1993).

Because the slope of log(MSD) *vs.* log(Δt) plots is not sufficient to discriminate between normal and abnormal diffusion, OMEGA implements a method developed by Ferrari et al. and based on the estimation of the Hurst exponent (Hurst *et al*, 1965; Hurst, 1951; Ferrari *et al*, 2001) to increase the accuracy of motion type prediction. This method, primarily referred to as Moment Scaling Spectrum (MSS) analysis, extends the study of the logarithmic scaling behavior with respect to Δt to moments of displacement ($\mu\nu$) other than the moment of displacement of order $\nu = 2$, which corresponds to the MSD (i.e., MSD = μ_2). Thus, a MSS graph is constructed plotting the values of $\mu\nu$ vs. the corresponding values of the logarithmic scaling factor (i.e., $\Upsilon\nu$) and the Slope of the MSS curve (SMSS) is used to discriminate between different motion modalities, where SMSS = 0.5 corresponds to normal diffusion, while values of SMSS $\neq$ 0.5 correspond to anomalous diffusive states (Supplemental Table II). In order to calculate the quantity of displacement, OMEGA calculates the generalized Observed Diffusion Constant of order $\nu = 2$ (ODC_2), which in case of a purely diffusive Brownian particle

coincides with D (Saxton & Jacobson, 1997).

Specifically, OMEGA calculates the values of $\mu\nu$ for ten different orders (ν= 1-10) as well as all corresponding $\Upsilon\nu$ and ODCν values, and reports them in tabular form. In addition, OMEGA reports values of ODC2 (i.e., henceforth referred to as ODC) calculated from the intercept of the linear regression of log-log (ODC2log) plots of MSD vs. Δt (Sbalzarini & Koumoutsakos, 2005), which is more robust in case of trajectories that differ significantly from free normal diffusion. Finally, the value of the slope of the log-log plot of MSD vs. Δt ($\Upsilon 2$), as well as the SMSS (also termed β) are reported as global estimates of the dynamic behavior of particles under study and made available in both graph and table format (Figures 4-8; Supplemental Figure 3; Supplemental Table I).

2.6.2. Motion type classification

Global motion analysis reduces whole trajectories to a series of individual measurements or features (Tables I and II). The combination of two or more of such features enables the representation of individual trajectories as points in n-dimensional phase space. In addition to representing a massive data reduction, this approach has the advantage of facilitating the classification of the mobility characteristics of multiple particles all at once without arbitrary selection. Thus, trajectories clustering in phase space are expected to have similar dynamic behavior and in turn correspond to similar functional states. An additional advantage of this method is that states described in this manner could be defined dynamically depending on individual scientific questions while at the same time could be the subject of standardization. At the time of writing, motion classification in OMEGA is based on the phase space of SMSS *vs.* ODC, which allows to quantify both the "speed" and the "freedom" of a group of moving objects independently, as previously described (Sbalzarini & Koumoutsakos, 2005; Schelhaas *et al*, 2008). The addition of further features to augment phase-space clustering, such as the Directional Change between subsequent displacement steps or measures of anisotropy (Huet *et al*, 2006) is easily implementable due to the generic nature of the underlying architecture and is planned for future releases.

Iterative motion type classification/segmentation workflow

As mentioned, motion type classification in OMEGA is based on the visual inspection of log-log plots describing the variation of either MSD or of moments of displacement of different order over increasing time intervals (Supplemental Table II). In the presence of motion type transitions within an individual trajectory (e.g. periods of confinement followed by normal diffusion; or periods of deterministic drift interspersed with bursts of directed motion), global quantitative measures that are averaged over the entire duration of the trajectory, such as ODC and SMSS, represent unreliable estimates of particle dynamics (Ewers *et al*, 2005; Helmuth *et al*, 2007). To obviate this hurdle, OMEGA implements an interactive pipeline for motion type classification (Figures 5 and 6; Supplemental Figure 3), which is based on the notion that the MSS plot carries information about the "self-similarity" of the motion under study (Ferrari *et al*, 2001). Specifically, if all moments in the spectrum scale linearly with order, then the MSS is a line and the motion is defined as "strongly self-similar"; conversely if the MSS curve is kinked or bent, the movement is classified as "weekly self-similar", indicating

the existence of transitions between different states.

All of these observations lead to a straightforward iterative workflow (Figure 5A): 1) after particle tracking, trajectories are subjected to MSS analysis. 2) If the resulting plot is observed to be bent, the trajectory can be iteratively subdivided into segments until all resulting segments produce a straight MSS line. 3) At this point, ODC and SMSS are estimated and each segment is plotted as a point in phase space. The position of each trajectory in phase space as described above reflects their dynamics and is used to assign trajectories to motion type classes whose frequency in the segment population can be estimated by drawing windows around clouds of points, and compared across experimental conditions by statistical analysis using third-party applications as a prerequisite for functional analysis.

As an example, uniform artificial trajectories of known mobility were generated using the custom-made *artificialTrajectories2* Matlab routine (Helmuth *et al*, 2007), imported into OMEGA using the Data Browser module and assigned the corresponding motion type label by using the OMEGA Trajectory Segmentation plugin (Supplemental Table II). After subjecting to diffusivity analysis using the corresponding OMEGA plugin, trajectories were classified on the basis of their measured ODC and SMSS, which resulted in excellent agreement with the ground-truth behavior (Figure 5B and Supplemental Figure 3).

In order to mimic iterative segmentation vs. classification, uniform artificial trajectories were merged to produce trajectories comprising five different motion types. This resulted in "bent" MSS curves, indicating the non-uniform nature of the overall process. When the mixed trajectory was subdivided in segments and each was analyzed individually, this gave rise to five independently linear MSS curves allowing each segment to be correctly classified independent of its neighbors (Figure 6).

2.6.3 Estimation of motion type classification error

In order to interpret and draw valid conclusions from analysis results, the error associated with each measurement or calculation has to be determined and its effect on downstream analysis steps (i.e., error propagation) has to be clearly understood. Despite the apparent truism of this statement, attention to error propagation in particle tracking has been limited (Sbalzarini, 2016). To address this issue, OMEGA incorporates both theoretical and empirical methods to estimate uncertainties associated with motion analysis results. Details of our error estimation procedure are described in an associated manuscript (Rigano *et al*), here we provide a short description of the method.

While linking errors are orthogonal to motion analysis and are addressed elsewhere (Tinevez *et al*, 2016), uncertainty associated with spot detection strongly affects the accuracy with which trajectories can be classified on the basis of their observed dynamic behavior (Ewers *et al*, 2005). In addition, even with infinitely precise and true positioning, trajectory measures are expected to display statistical uncertainty because of finite trajectory lengths. These finite-data uncertainties diminish as the number of points that are detected as part of each trajectory increases. In addition to these well-known sources of error (i.e., position and sampling), we also

determined that the quantity of displacement as well as the freedom of motion of moving particles (i.e., ODC and SMSS respectively) affect motion type estimation uncertainty.

When image quality is low (i.e., low SNR), the verisimilitude of positioning estimates might be as low as to make it difficult to distinguish between actual movement and apparent positional shifts arising from both systematic and random localization errors. This is particularly problematic for random walks, which can be discriminated from sub-diffusing particles and even from stationary particles only when their ODC is large enough to cause particle motion larger than the localization uncertainly (Martin *et al*, 2002). Based on these premises, given the observed image quality it is possible to define a "limit of detection" below which ODC values can be considered meaningless. For this purpose we employ the global error model described by Martin et al. (Martin *et al*, 2002) to calculate Minimum Detectable ODC of order 2 ($ODC_{2\ MinDet}$) values as a function of image quality and detection (see Supplemental Information 1). Once calculated, $ODC_{2\ MinDet}$ is reported in both tabular and graphical form (Figures 7 and 8). This threshold can be used to exclude from subsequent analyses steps trajectories whose global ODC value is too low to be meaningfully distinguished from noise.

Despite significant advances (Martin *et al*, 2002; Gloter & Hoffmann, 2007) the effect of positional uncertainty and sample size on motion type estimates remain difficult to theoretically predict. Thus, we reasoned that a better approach would be to empirically estimate the uncertainty associated with each ODC and SMSS measurement (i.e., local error analysis). For this purpose, we developed a numerical method, based on the Monte Carlo simulation of artificial trajectories, whose true position with respect to the imaging system, rate of displacement (i.e., ODC), freedom of movement (i.e., SMSS) and length and are fully known (Rigano *et al*). After simulating the effect of positional error on these "ground truth" trajectories under different image quality contexts (i.e., SNR), ODC and SMSS are back-computed from the resulting "noisy" trajectories and the comparison between input and output values is used to estimate the uncertainty associated with motion type estimation as a function of expected motion characteristics (i.e., ODC and SMSS), motion duration (i.e., trajectory length L) and image quality (i.e., SNR). Using this information, the empirically generated four dimensional matrices relating L, SNR, ODC and SMSS values with expected values of ODC and SMSS, are interrogated by linear interpolation to obtain the uncertainty value associated with each trajectory under study. In turn, these ODC and SMSS uncertainty values are reported both in tabular format and as confidence intervals on two-dimensional ODC vs. SMSS scatter plots, which forms the basis for motion type classification in OMEGA.

2.7 Data browser, storage and export: trajectory data management and dissemination

The Data Browser is the main data management gateway for OMEGA (Figure 2-12; Supplemental Information 1) and it provides an intuitive interface that allows users to navigate across the entire data and metadata chain from images to trajectories, segments, tracking measures, and motion analysis uncertainties. It

facilitates the execution of four main processes each associated with its specific data path: 1) interactively navigate and display analysis output already present in OMEGA; 2) import pre-computed orphaned trajectories; 3) import previously stored analysis results associated with available images or trajectories; 4) save selected analysis results to database or export it for downstream analysis in third party applications.

2.7.1 Data Browser

At each step of the analysis workflow (Figures 1 and 4), the user can decide to compare results obtained using different parameter settings. Consequently, each set of results (i.e., detected particles, trajectories, edited trajectories, segments etc.) can be considered a branching point the dependence tree of possible result datasets. Consistent with this hierarchical structure, the Data Browser module facilitates the interactive display of dynamic lists that are populated with the analysis children of any selected data element (Figure 2-12; Supplemental Information 1). These resulting trees are presented to the user using the familiar Column View interface, where relevant metadata and result summaries are displayed at the bottom of each column to facilitate the identification of the desired data path. Additionally, to reduce work space clutter, results that at any point of time are not of immediate interest can be temporarily hidden from the view by unchecking their selection mark. Once identified and selected, results branching off a given element can be imported from the OMEGA database, displayed in parallel on all concurrently opened windows (e.g. Trajectory Browser, Side Bar and Tracking Measures), saved to the database, or exported for use on third-party applications.

2.7.2 Data storage and data provenance

Information describing the "origin" and "lineage" of data is essential for scientists to be able to correctly interpret image analysis results. Such information is often referred to as describing "data provenance" and can be conceptualized as metadata capable of answering key questions describing manipulation events occurring during the data lifecycle (Ram & Liu, 2009). To facilitate tracking the provenance of data, comparing results across laboratories and reproducibility, OMEGA bridges between dedicated image servers to retrieve image data and metadata (Goldberg *et al*, 2005) and dedicated results databases to mine and store analytical output. OMEGA stores the entire particle tracking and analysis results data chain in a dedicated relational data-hub whose schema recapitulates our recently proposed Minimum Information About Particle Tracking Experiments (MIAPTE) guidelines (Rigano & Strambio-De-Castillia, 2017). The use of MIAPTE facilitates management of data quality, particle tracking, motion analysis and error estimation results and facilitates meaningful comparison and reproduction of results obtained at different moments in time and from different laboratories (Figure 1C; Rigano & Strambio-De-Castillia, 2017). This database stores trajectories, analysis parameters, motion analysis results, and associated uncertainties and links all of this information with the source image data and metadata. All access to the OMEGA database is mediated by the OMEGA client making its presence transparent to the user.

2.7.3 Result data export

In addition to being able to store structured data in the OMEGA database, the user can alternatively chose to export any portion of the tracking and motion analysis results to file for third-party secondary analysis. In case of "orphaned trajectories", export to file is the only available option for results storage. This function is available in the Data Browser where the user can choose to export specific results or entire particle tracking and trajectory analysis sessions including all analysis definition metadata to facilitate downstream statistical analysis using third party applications (i.e., R or Matlab), data exchange with other researchers, meta-analysis and reproduction of results.

3 – Example use-cases and applications

We present here two use cases to illustrate OMEGA functionality. The first test case takes advantage of simulated image datasets that were produced to directly compare different MPT algorithms (Chenouard *et al*, 2014); scenario IV, infecting viral particles; SNR = 7; low particle density). As expected, when images were subjected to MPT within OMEGA, most trajectories displayed a stretched out appearance with occasional direction transitions, mimicking a condition where most particles display the tendency of "flying" over long distances in a particular direction (i.e., Lévy flights; Levy, 1937), such as what is observed in active motion (Figure 7). When trajectories were manually inspected, most appeared to be correct. However, trajectory nr. 424 appeared to be composed of two erroneously linked trajectories (Figure 7B), consistent with the observed "kinked" shape of the MSS curve (Figure 7C, bottom left, *arrowhead*). The use of the Trajectory Editing tool (Figure 7 A and D) allowed to split this trajectory in two individual trajectories, 424.1 and 424.2 (Figure 7E and F). While trajectory 424.1 gave rise to a straight MSS curve for trajectory consistent with the identification of a uniformly mobile particle, a similar analysis of 424.2 produced a bent curve indicating that such trajectory is apparently produced by a particle whose mobility is non self-similar and would require segmentation (Figure 7F). After trajectory editing, all resulting trajectories were subjected to diffusivity analysis (Figure 7G-K). The results of such analysis were consistent with the trajectories displaying directed motion, as indicated by the clustering of trajectories in the top quadrant of the phase space (Figure 7J, *red circled area*) as well as the prevalence of SMSS values close to 1 (Figure 7K). In particular trajectories nr. 27 and 32, as well as edited trajectory nr. 424.1, displayed a straight MSS curve suggesting uniform mobility and validating the ODC and SMSS values estimations calculated in OMEGA. It should be noted that the ODC and SMSS estimation errors we observed are consistent with a high SNR level and relatively short trajectories (i.e., relatively small ODC error and relatively high SMSS error).

The second OMEGA test case is a real-life example provided by the Hunter's lab (Clark *et al*, 2013; Pereira *et al*, 2012; Pereira *et al*, 2014). In this example, CMMT rhesus macaques (*Macaca mulatta)* mammary tumor cells chronically infected with Mason-Pfizer Monkey Virus (M-PMV), a D-type retrovirus, were co-transfected with a plasmid expressing a codon-optimized GFP-tagged variant of the M-PMV Gag precursor polyprotein

alongside one expressing mCherry-Tubulin (i.e., a microtubule subunit). Seven hours post-transfection cells were either mock-treated (Figure 8, *Untreated*) or treated with the microtubule polymerization inhibitor Nocodazole (Figure 8, *Treated*) for 1 hour prior to live imaging to observe the assembly of viral particles and their trafficking towards the plasma membrane. Example images were first imported into OMERO and then loaded into OMEGA for MPT (Figure 8A and B). Trajectories were examined using the OMEGA DTM plugin and a subset of trajectories displaying uniform mobility as indicated by a straight MSS graph, were assigned a specific motion type as indicated by the position of the line on the MSS plot. In Untreated cells, most trajectories were found to display a sub-diffusive behavior (Figure 8B and C, *fuchsia*) with a minority of viral particles displaying clearly diffusive and super-diffusive mobility (Figure 8B and C, *blue* and *purple* respectively). An example super-diffusive trajectory (i.e., *purple*) is displayed in the bottom insert in panel 7B. As expected, when cells were treated with Nocodazole, the global intracellular mobility of viral particles was dramatically reduced as testified both by the overall collapse of resulting trajectories (Figure C and D, compare top left panels) and by the clustering of viral trajectories in ODC vs. SMSS phase space (Figure 8C and D, right panels, compare the grey area vs. the red area). While the biggest effect was observed on ODC values, which drastically diminished, a significant effect was also observed on the MSS behavior as shown by comparing the resulting SMSS distributions in Untreated vs. Treated cells (Figure 8C and D, bottom left panels; Figure 8E). Of note, this analysis took a total of 5 minutes and required no manual tracking testifying the advantage of using OMEGA for increasing the throughput of systematic motion analysis experiments to higher levels than allowed by the use of individual and analysis tools.

4 – Discussion

Despite tremendous improvements in space and time resolution of modern microscopic techniques, the translation of such advances towards increased understanding of intracellular particle movement has proceeded at a significantly slower pace. This state of affairs cannot be circumvented without the development of a collaborative infrastructure that allows the direct interaction of experimental scientists that have a deep understanding of the biological system under study, with image analysis experts, mathematicians, statisticians, algorithm developers and software engineers that can help make sense of the data. Such collaborations are increasingly developed at the local scale (i.e., individual well-funded laboratories and inter-institutional ad-hoc collaborations). However, in order to facilitate the paradigm shift that is required for a truly systematic approach to intracellular trafficking, one that is capable of integrating genomics, transcriptomics, proteomics and functional data, it is necessary to develop a virtual "table" where all required expertise can convene across time, space, experimental systems and contexts to bring about substantial progress towards the fundamental understanding of the system as a whole. In order to provide a significant contribution towards this goal we have developed OMEGA. This tool has the following important characteristics:

1. OMEGA focuses uniquely on the analysis workflow required for multiple particle tracking and motion analysis.
2. OMEGA can serve as a testing ground for the development of analytical and data management standards that can be then expanded to other fields.
3. OMEGA provides a shared framework that can be used across expertise level by all the stakeholders that need to be involved in particle tracking.
4. OMEGA provides explicit and open-access support for uncertainty estimation and for the evaluation of how such error propagates through the analysis routine. This is arguably the first essential step towards true data standardization and data sharing.

Open-source, bioimage informatics initiatives largely focus on the production of general tools to address several individual analytical needs with much less emphasis on integration of data management and error propagation (Table I; Tinevez *et al*, 2016; de Chaumont *et al*, 2012; Cardona & Tomancak, 2012). We reasoned that a better approach would be to horizontally tackle a very limited set of biological questions and address them holistically from data acquisition to results interpretation. As a test case, we decided to study the dynamic behavior of retroviral viral particles during the initial phases of the viral life cycle. We reasoned that this approach would have several advantages. Firstly, retrovirus cell biology is well within our realm of expertise (Xu *et al*, 2013; Pertel *et al*, 2011a; 2011b; Sokolskaja *et al*, 2010; Neagu *et al*, 2009; Sebastian *et al*, 2009; Strambio-De-Castillia & Hunter, 1992) and it is a relatively technology-poor field, with well-documented and urgent scientific and quantitative analysis needs, and consequent opportunity for development. Second, MPT and motion analysis entail a well-defined series of analytical steps, which currently are not well integrated among one another and lack, metadata and procedure standardization as well as error estimation. Third, initiatives to foster community efforts for the improvement of MPT and motion analysis tasks are well underway (Chenouard *et al*, 2014) potentially facilitating further collaboration. Finally, this approach could serve as proof-of-principle for the maturation of inter-disciplinary collaborations between experimental and computational scientists that could be extended to other image analysis tasks.

To enhance usability by scientists OMEGA relies on graphically supported user interactions. To improve interoperability and use by developer-users, the platform relies on a solid modular architecture, with defined and well-documented programming interfaces. To facilitate data sharing and reproducibility, OMEGA assigns a Universally Unique Identifier (UUID) to each data element, and complies with the existing OME-TIFF image metadata definition standard as well as our newly proposed MIAPTE guidelines. Finally, by directly addressing issues of error propagation and data provenance, and by relying on semantic data models to record tracking results and analysis-definition metadata (Goldberg *et al*, 2005; Rigano & Strambio-De-Castillia, 2017), OMEGA lays the ground for the development of analytical standards for particle tracking. Such standards are a prerequisite for data reproducibility, reusability and transferability between research groups.

5 – Conclusions

OMEGA is a novel cross-platform data management system for particle tracking experiments. OMEGA is freely available, flexible and easily extensible. It links upstream image data and metadata with downstream motion analysis tools, it automates data handling, processing, quality monitoring and interpretation, ultimately facilitating the comparison of image analysis results, of data analysis routines and of uncertainty quantification both inside and across laboratories.

OMEGA's intuitive interface facilitates data selection, data import, analysis and reporting of analysis results and uncertainties. Data can be exported for use in third party tools and across laboratories laying the foundation for the meta-analysis of data generated by multiple users and making it possible, for example, to compare the effect of specific treatments on particle motion across different experimental systems.

In conclusion, OMEGA facilitates the cooperation of all players whose role is required to understand complex biological systems: biological scientists, image analysis experts, algorithm developers, statisticians and software engineers. OMEGA is developed following a modern open development paradigm, which allows the entire bioimage informatics community to participate in its development. Thus, OMEGA facilitates the process of incorporating both novel and already available tools to build integrated data processing and analysis pipelines for quantitative, real-time, sub-cellular particle tracking. We hope that OMEGA will significantly contribute to the development of universal tools that can be used across multiple scientific questions, model systems and experimental contexts.

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Supplemental material

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Supplemental Figures

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Table I **Overview of currently available integrated software tools supporting particle tracking and motion analysis workflows**

Software tool	Data management								Analysis												Quality control			
Features	Experimental design annotation	Image loading from repository	Trajectory import	Data management and storage	Utilization of minumum information reporting guidelines	Data chain browsing	Export of fully annotated data cascade	Graphical presentation of results	Particle detection	Particle linking	Manual tracking	Bach processing	Results inspection and validation	Trajectory manager	Trajectory editing	Trajectory segmentation	Motion analysis (Intenisty, Mobility, Velocity)	Diffusivity analysis	Anisotripy analysis	Motion type classification	Image quality assessment	Estimation of particle detection error	Estimation of particle linking error	Estimation of motion type classification error
ICY (deChaumont et al., 2012)	n.a.	n.a.	+	n.a.	n.a.	n.a.	n.a.	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	+ (M, V)	+ (MSD)	n.a.	n.a.	n.a.	n.a.	+	n.a.
ImageJ TrackMate/TrajClassifier plugins (Tinevez et al., 2016)	n.a.	+	+	n.a.	n.a.	n.a.	n.a.	+	+	+	+	n.a.	+	+	+	+ (auto-matic)	+ (I)	n.a.	n.a.	+ (Wagner et al., 2017)	n.a.	n.a.	n.a.	n.a.
ImageJ MOSAIC Particle Tracker 2D/3D plugin (^)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+ (MSD and MSS)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
OMEGA	+ (OMERO)	+ (OMERO with full metadata)	+	+	+ (OME-XML, MIAPTE)	+	+ (Images to motion analysis results with full metadata)	+	+	+	n.a.	+	+	+	+	+ (iterative manual)	+ (I, M and V)	+ (MSD and MSS)	+	+ (Ewers et al., 2005)	+	+	n.a.	+

Legend

(^) MOSAICsuite (https://imagej.net/MOSAICsuite)

Figures

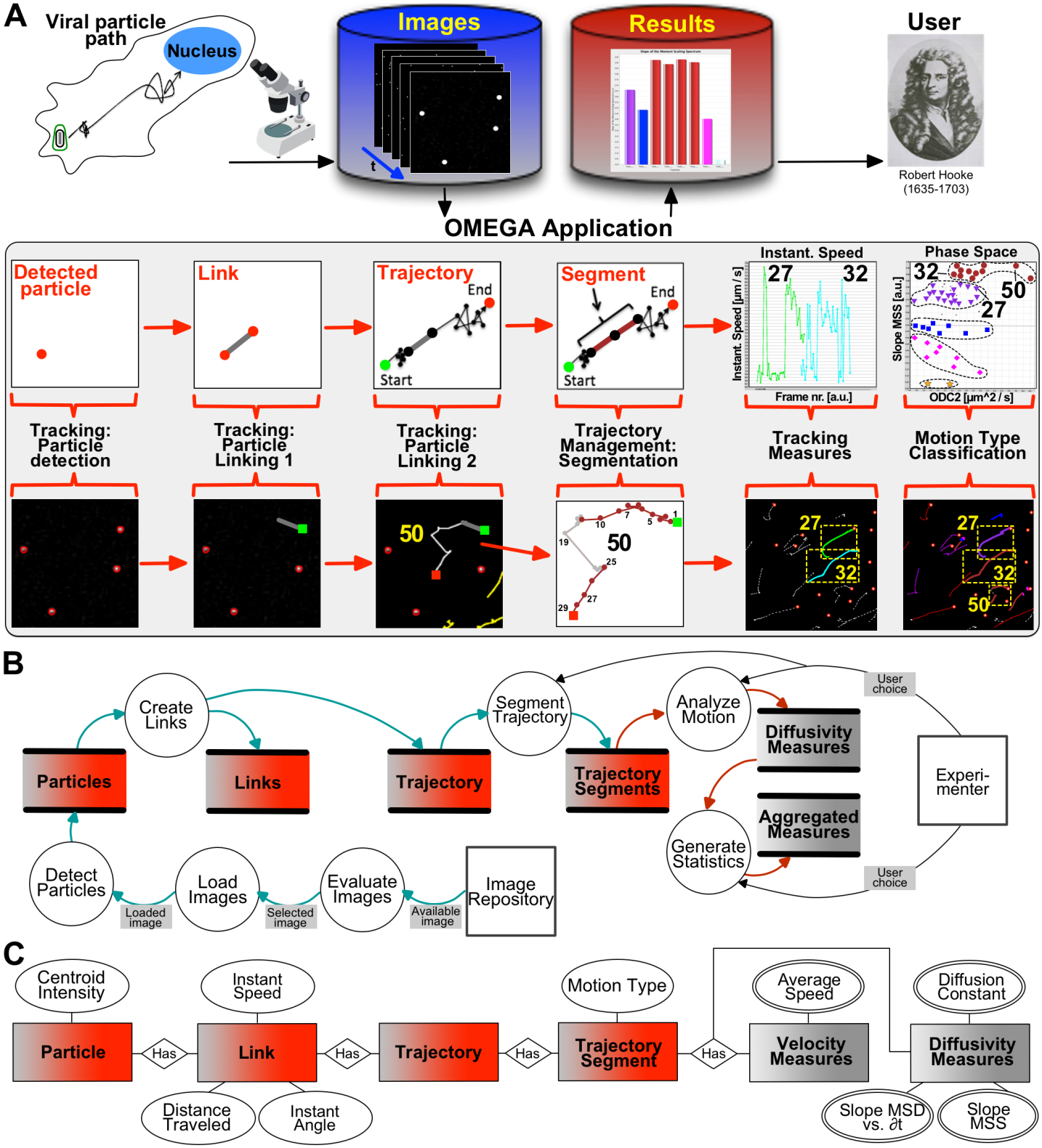
A
Viral particle path
Nucleus
Images
Results
User
Robert Hooke (1635-1703)
OMEGA Application
Detected particle
Link
Trajectory
End
Start
Segment
Instant. Speed
Phase Space
Instant. Speed [µm / s]
Frame nr. [a.u.]
Slope MSS [a.u.]
ODC2 [µm^2 / s]
Tracking: Particle detection
Tracking: Particle Linking 1
Tracking: Particle Linking 2
Trajectory Management: Segmentation
Tracking Measures
Motion Type Classification
B
Create Links
Particles
Links
Trajectory
Segment Trajectory
Trajectory Segments
Analyze Motion
Diffusivity Measures
Aggregated Measures
Generate Statistics
User choice
Experi-menter
Detect Particles
Load Images
Evaluate Images
Image Repository
Loaded image
Selected image
Available image
C
Centroid Intensity
Instant Speed
Motion Type
Average Speed
Diffusion Constant
Particle
Has
Link
Trajectory
Trajectory Segment
Velocity Measures
Diffusivity Measures
Distance Traveled
Instant Angle
Slope MSD vs. ∂t
Slope MSS

Figure 1:

OMEGA imports images stored in OMERO executes a complete viral particle tracking and motion analysis workflow and manages results in the OMEGA results repository on the basis of a MIAPTE compliant data model. Schematic diagram depicting the system context in which OMEGA operates and the workflow required for the estimation of the sub-cellular trajectories followed by diffraction-limited intra-cellular viral particles and the computation of biologically meaningful measures from particles coordinates. A) Images are acquired using any available microscope and imported into an available OMERO database. OMEGA imports images using the Image Data Browser plugin and subjects them to MPT in two independent steps using the Particle Detection and the Particle Linking plugins. As needed individual trajectories (in the example trajectory nr. ***50***) can be subdivided in uniform segments using the interactive Trajectory Segmentation plugin. In the example, trajectory nr. **50** was subdivided in three segments two of which were assigned the Directed motion type (*maroon*) and the third one was left un-assigned (*grey*)**.** In addition, all other trajectories which appeared to be uniform in nature were assigned the color corresponding to the predicted motion type depending on the observed slope of the MSS curve (*grey*, unassigned; *yellow*, confined; fuchsia, sub-diffusive; *blue*, diffusive; *purple*, super-diffusive; *maroon*, directed). Trajectories were then subjected to motion analysis using the VTM and the DTM plugins. Instantaneous Speed results for trajectory nr. **27** and **32** and D vs. SMSS Phase space results for all trajectories are displayed. The position of spots representing trajectory nr. **27, 32** and **50** are indicated. B) The path taken by data across the workflow indicated in panel A is represented using the data flow diagram (DFD) formalism. Here circles represent processes that transform data, arrows represent data in motion, arrow labels represent specific packages of data being moved, double lined-rectangles represent data at rest (i.e. data stores) and squares represent entities (i.e. Experimenter(s) and external data repositories) that interact with the system from the outside. C) In order to ensure the preservation of data provenance links, OMEGA utilizes a relational database whose model in based on our recently proposed Minimum Information About Particle Tracking (MIAPTE) guidelines. Depicted here is an Entity Relationship diagram representing the corresponding OME-XML (*blue*) MIAPTE (*red*, particle data; *grey*, analysis data) elements utilized to capture data pertaining to each step of the data-flow.

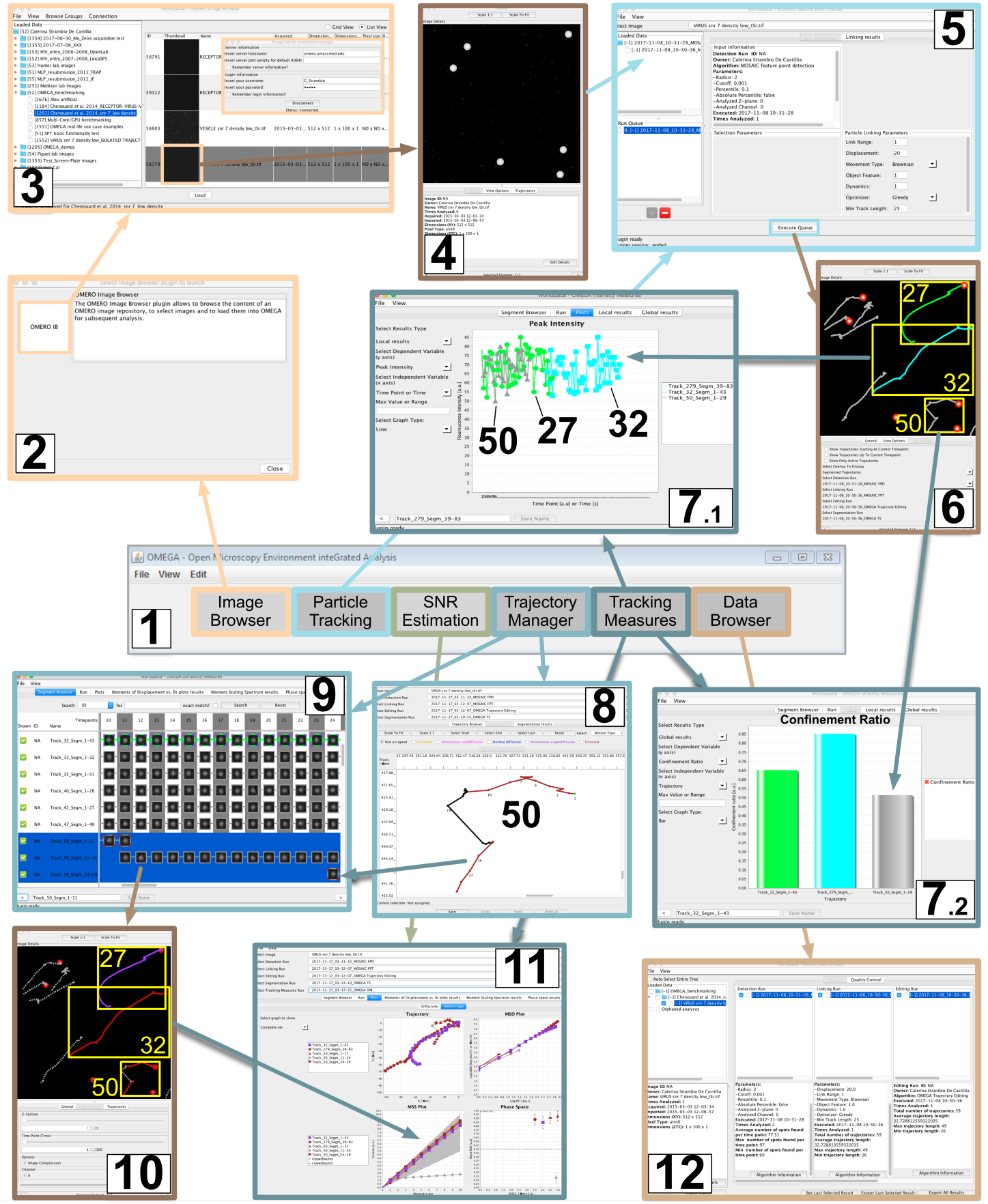
3
4
5
2
7.1
6
1
Image Browser
Particle Tracking
SNR Estimation
Trajectory Manager
Tracking Measures
Data Browser
9
8
7.2
10
11
12
Peak Intensity
Confinement Ratio
Trajectory
MSD Plot
MSS Plot
Phase Space

Figure 2:

OMEGA for users: the graphical user interface. Upon starting the OMEGA application the user can access available plugins using the top-bar **(1)**. After opening the Image Browser launch-pad **(2**) the user can launch the OMERO Image Browser plugin to select and load **(3)** one or more images of interest for inspection in the sidebar viewer **(4)**. After defining different paired Particle Detection and Particle Linking **(5)** runs the resulting spots and trajectories can be visualized as overlays via the sidebar image viewer **(6)**. Trajectories of interest (in the example trajectory nr. **27, 32** and **50**, as indicated here and in Figure 1**)** can be highlighted with individual colors (in the example trajectory 27, *green* and trajectory 32, *turquoise*) in order to facilitate their identification in all available views. As an example, after executing the Intensity **(7.1)** and Mobility **(7.2)** Tracking Measures plugins, results obtained with selected trajectory nr. **27, 32, 50** can be plotted using the same colors used on the sidebar to facilitate results comparison and interpretation. In case individual trajectories appear to be non-self similar as often observed with intracellular viral particles (viz. in this case trajectory nr. **50**), they can be subdivided into two or more individual segments of uniform motion type using the interactive graphical user interface provided as part of the Trajectory Segmentation plugin **(8)**. In the example, trajectory nr. **50** was subdivided in three segments: segments 1-11 and 24-29, which were assigned the directed motion type (*maroon*) and segment 11-14, which was left unassigned (*grey*). As a result of this assignment, when displaying trajectories that have undergone segmentation on the Trajectory Browser **(9)**, they appear split in individual sections each possessing two colors: the color that was assigned by the user to the unsegmented trajectory is displayed as the main square color (*grey,* in the highlighted example referring to trajectory nr. **50**); the color that corresponds to the user-assigned motion type instead appears as the secondary color shown only at the square vertexes (*maroon*, in the highlighted example referring to trajectory nr. **50**). In addition, after segmentation each portion of the trajectory is displayed on the Trajectory Segmentation window, the side bar **(10)** and each of the Tracking Measures plot windows **(11)** using the user-assigned motion type color. In addition in order to facilitate user interpretation of the results, portions of a segmented trajectory that were not assigned a specific motion type are now displayed on the sidebar as dashed lines making it clear to the user that the segmentation process has taken place. At each step of the particle tracking and motion analysis workflow the user can maintain a clear picture of all available results stemming from different image-analysis data-flows using the OMEGA Data Brower **(12).** An additional advantage of this plugin is that it can be used to compare results from different runs as well as load results obtained from previous OMEGA sessions for further investigation.

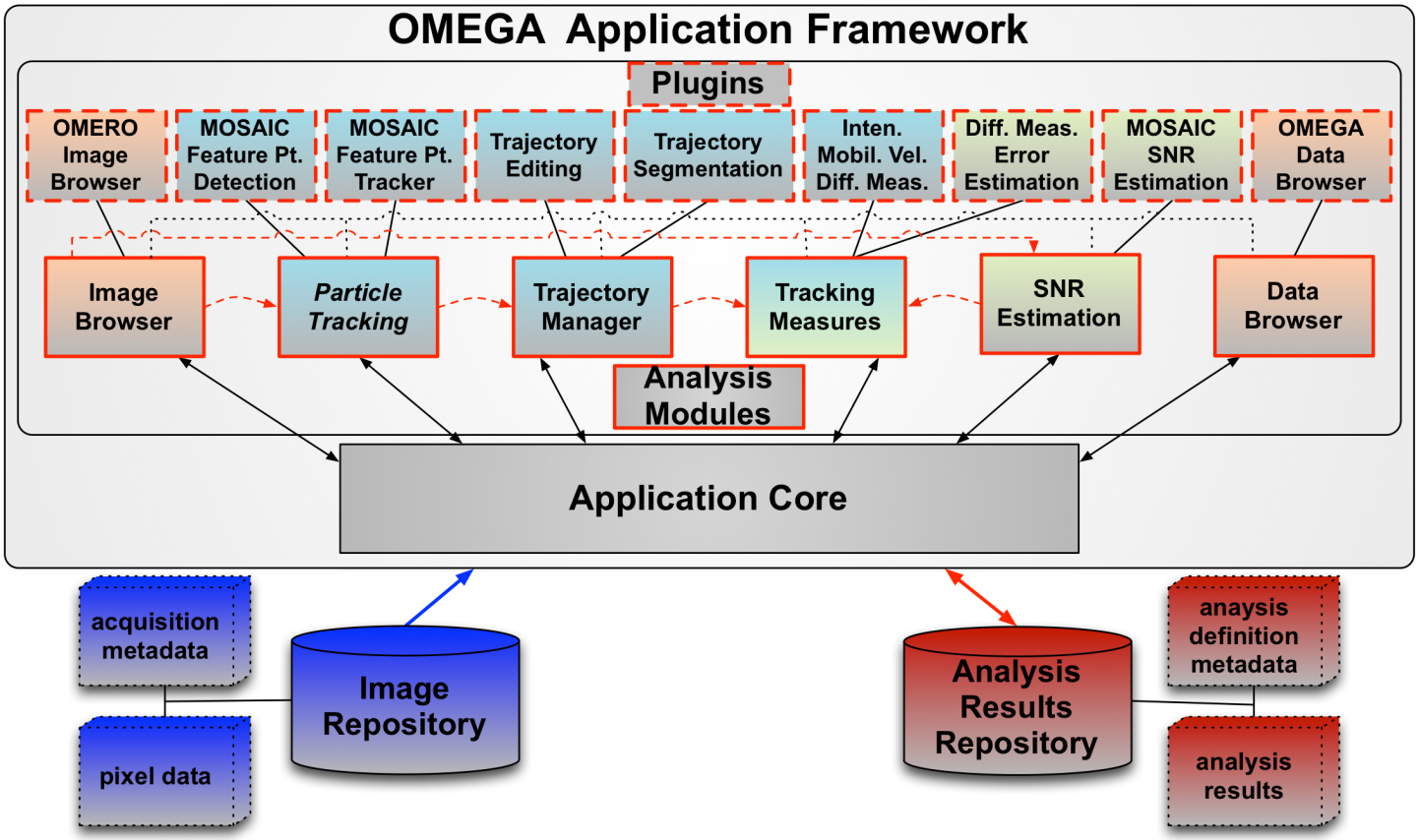

Figure 3:

OMEGA's modular software architecture. The framework of the OMEGA application contains an Application Core, which drives all processes and interacts with the Image (*blue elements*) and the Analysis Results (*red elements*) data stores; and an Application Modules component, which is responsible of carrying out OMEGA particle tracking and motion analysis core functionality. The Application Core contains all main sub-components including the event-driven logic driving all communication between the core and the analysis modules. The core also contains all GUI sub-components including the top menu bar, the side bar and the workspace, the analysis results repository preferences window, the module launcher dialog, which is opened every time users click on a module button on the top menu bar, and the plugin information dialog, which is opened when clicking on the additional information button of a specific plugin. The Application Modules component, contains all main functional logic in OMEGA and is organized around six modular element types: 1) Image browser; 2) Particle tracking; 3) Trajectory manager: 4) Tracking measures; 5) SNR estimation; and 6) Data Browser. Each of these module types is responsible for the execution of one or more interchangeable plugins, which in turn are responsible for specific steps of the analysis pipeline. Currently, OMEGA ships with a set of eight Analysis (*blue boxes*), two Quality Control (*green boxes*) and two Data Management plugins (*orange boxes*). The Analysis plugins are: 1) MOSAIC Feature Point Detection; 2) MOSAIC Feature Point Tracker; 3) OMEGA Trajectory Editing; 4) OMEGA Trajectory Segmentation; and 5-8) OMEGA ITM, MTM, VTM and DTM. The Quality Control plugins are: 1) MOSAIC SNR Estimation; and 2) OMEGA Diffusivity Measure Error Estimation. The Data Management plugins are: 1) OMERO Image browser; and 2) OMEGA Data browser.

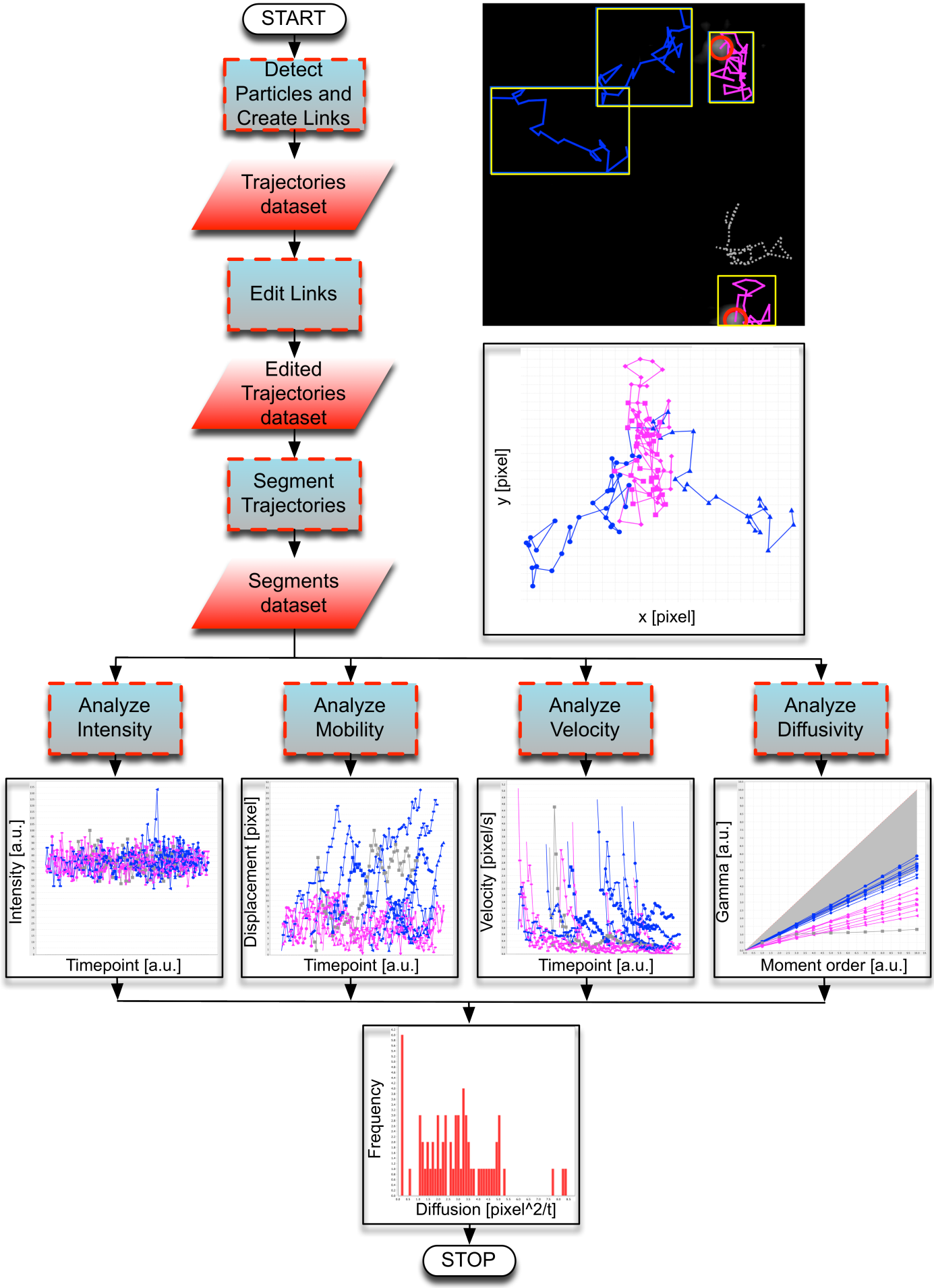
START
Detect Particles and Create Links
Trajectories dataset
Edit Links
Edited Trajectories dataset
Segment Trajectories
Segments dataset
y [pixel]
x [pixel]
Analyze Intensity
Analyze Mobility
Analyze Velocity
Analyze Diffusivity
Intensity [a.u.]
Timepoint [a.u.]
Displacement [pixel]
Timepoint [a.u.]
Velocity [pixel/s]
Timepoint [a.u.]
Gamma [a.u.]
Moment order [a.u.]
Frequency
Diffusion [pixel^2/t]
STOP

Figure 4:

Motion analysis workflow in OMEGA. Schematic representation of the motion analysis workflow implemented in OMEGA using a flow-chart diagram formalism. After particle detection and linking, when appropriate the resulting dataset of trajectories is subjected to link-editing and trajectory segmentation to produce uniform trajectory segments. Segments are then analyzed using one or more of the available OMEGA Tracking Measures plugins. Finally, frequency distributions can be computed or data exported for more extensive statistical analysis using third party applications.

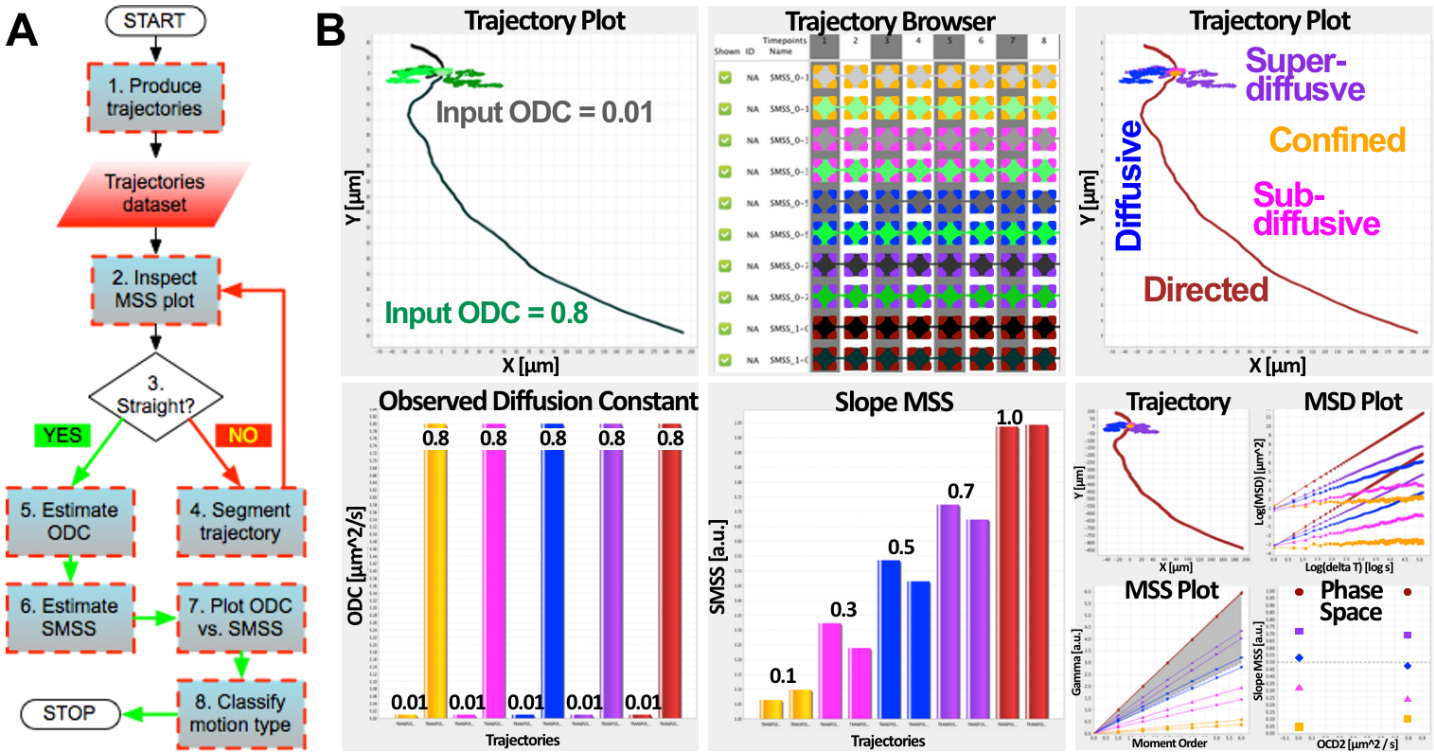

Figure 5

Motion type segmentation and classification in OMEGA. A) Flow-chart diagram depicting the iterative and interactive OMEGA motion type classification workflow. The workflow starts with the production of trajectories by particle detection and linking (Step 1). Then for each trajectory of interest in the dataset the workflow continues as follows: Step 2) Perform MSS analysis (Ferrari *et al*, 2001) and inspect the shape of each MSS plot. Step 3) If the plot appears bent, subject the trajectory of interest to Step 4, Else, continue to Step 5. Step 4) Perform Trajectory Segmentation and start again from Step 2. Step 5) Estimate ODC. Step 6) Estimate SMSS. Step 7) Plot each trajectory on ODC vs. SMSS phase space. Step 8) Use the position of each trajectory on phase space to classify motion. B) Classification example using uniform artificial trajectories of known mobility characteristics. Ten self-similar artificial trajectories of known mobility were generated using our *artificialTrajectories2* MatLab algorithm (Supplemental Information 1). After importing into OMEGA (top left) using the Data Browser data importer, they were first arbitrarily colored (i.e. shades of grey and green) and then assigned the motion type label corresponding to each expected motion type by using the Trajectory Segmentation plugin (top middle and right). Finally they were subjected to motion analysis using the DTM plugin (bottom row). Observed ODC (bottom left and right) and SMSS (bottom middle and right) numerical quantities and plot shapes were in excelled agreement with the corresponding indicated expected values. The position of each trajectory on the Phase Space plot was consistent with the expected motion behavior (bottom right). Input ODC values as indicated: 0.01 and 0.8 Input SMSS values as indicated: 0.1, 0.3, 0.5, 0.7 and 1.0. Motion types color codes: *yellow*, confined; fuchsia, sub-diffusive; *blue*, diffusive; *purple*, super-diffusive; *maroon*, directed (Supplemental Table II).

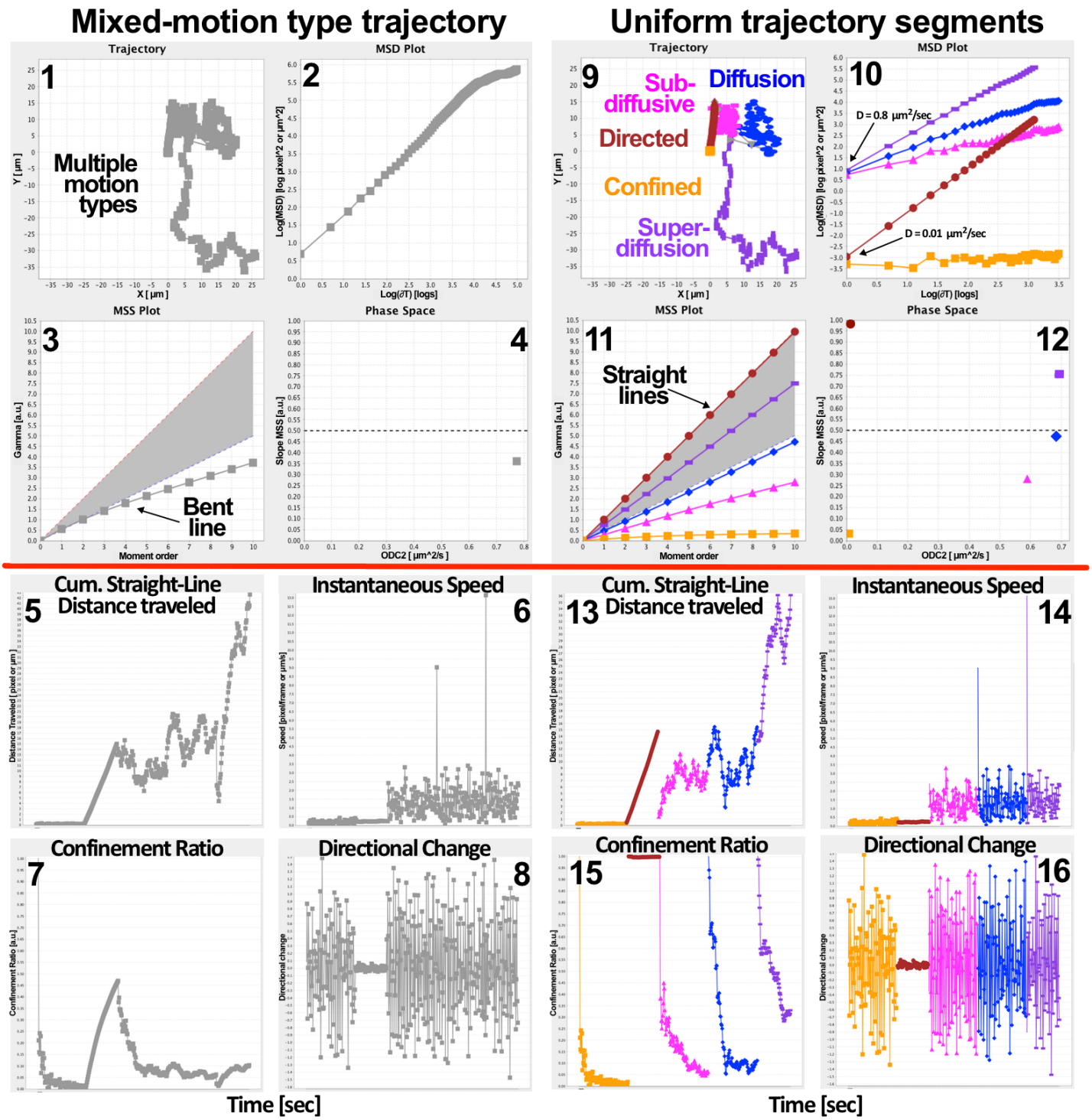
Mixed-motion type trajectory
Uniform trajectory segments
Trajectory
MSD Plot
MSS Plot
Phase Space
Multiple motion types
Bent line
Sub-diffusive
Diffusion
Directed
Confined
Super-diffusion
D = 0.8 μm²/sec
D = 0.01 μm²/sec
Straight lines
Cum. Straight-Line Distance traveled
Instantaneous Speed
Confinement Ratio
Directional Change
Time [sec]
Time [sec]

Figure 6

Segmentation and classification example using non-uniform artificial trajectories. Top: In order to mimic iterative segmentation followed by motion type classification, five of the uniform artificial trajectories of known ODC and SMSS described in B were merged to produce a single trajectory of mixed mobility (Mixed-motion type trajectory). When subjected to the Ewers motion type classification method (Ewers et al. 2005) implemented in the OMEGA DTM plugin (1 - 4), the mixed trajectory gave rise to a clearly "bent" MSS curve indicating the non-uniform nature of the process (3). In this context, both the calculated ODC (2 and 4) and SMSS values (3 and 4) represent averages values over the length of the trajectory and are therefore non-reliable. In order to obviate this obstacle and correctly classify each motion type component, the mixed trajectory was subdivided in segments using the OMEGA Trajectory Segmentation plugin and each segment was analyzed individually (Uniform trajectory segments). As can be clearly observed (9-12), this gave rise to five independent linear MSS curves (11) indicating that the trajectory had been correctly subdivided in uniform segments and allowing each segment to be correctly analyzed and classified independently from its neighbors (12). Bottom: The mixed-type artificial trajectory was subjected to motion analysis using the MTM and VTM plugins, before (Mixed-motion type trajectory) and after (Uniform trajectory segments) segmentation using the OMEGA Trajectory Segmentation plugin. When Straight-line Distance Travelled, Straight-line Speed, Confinement Ratio and Directional Change were plotted along each trajectory as a function of time, the resulting graphs reflected the presence of different motion components along the length of the full trajectory, which were clearly highlighted after subdivision into individual segments.

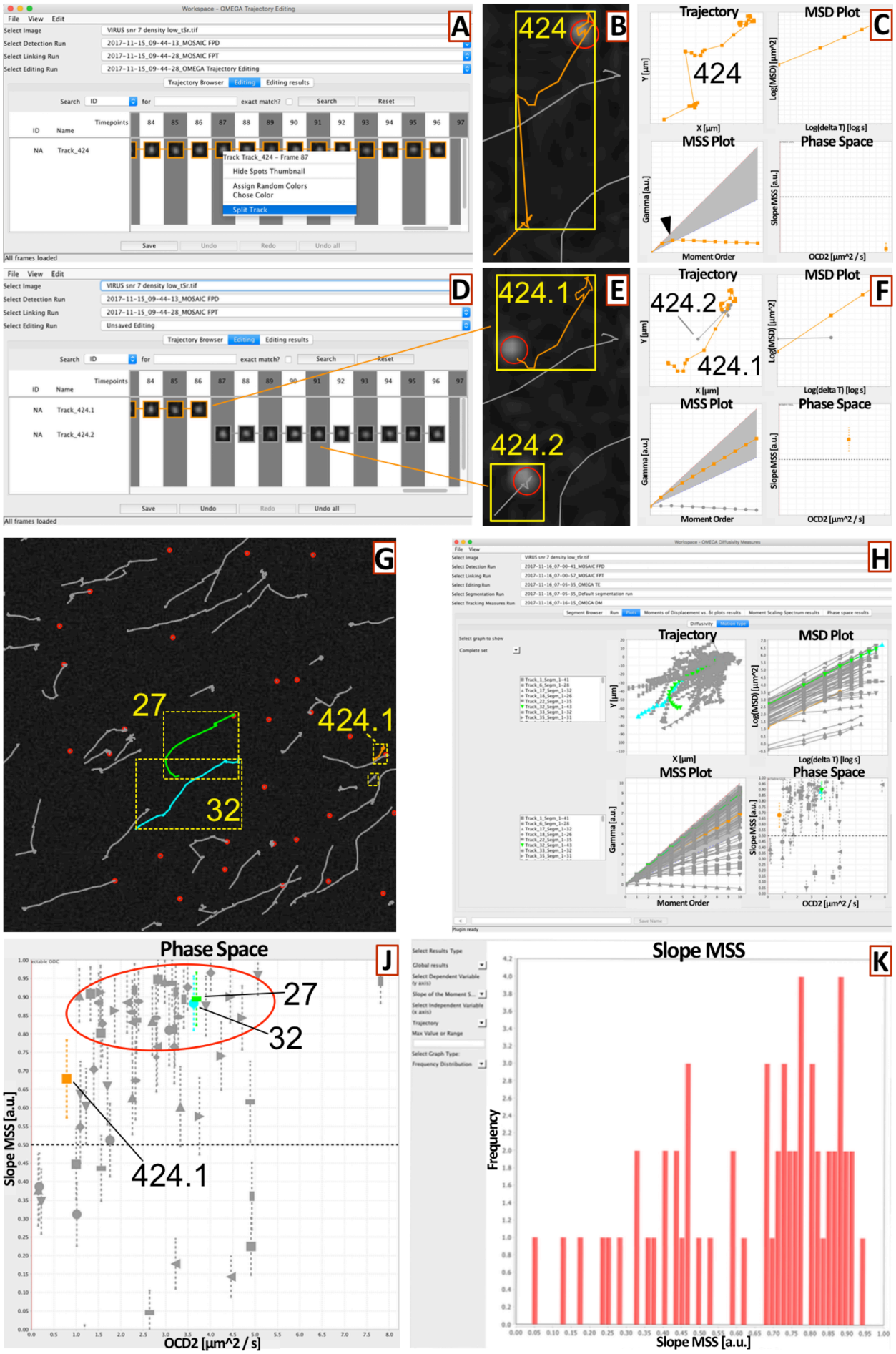
A
B
C
D
E
F
G
H
J
K
Workspace - OMEGA Trajectory Editing
Select Image
Select Detection Run
Select Linking Run
Select Editing Run
Trajectory Browser
Editing
Editing results
Search
Reset
Save
Undo
Redo
Undo all
All frames loaded
Track_424
Hide Spots Thumbnail
Assign Random Colors
Chose Color
Split Track
Unsaved Editing
Track_424.1
Track_424.2
424
424.1
424.2
27
32
Trajectory
MSD Plot
MSS Plot
Phase Space
X [µm]
Y [µm]
Log(delta T) [log s]
Log(MSD) [µm^2]
Moment Order
Gamma [a.u.]
OCD2 [µm^2 / s]
Slope MSS [a.u.]
Frequency

Figure 7

OMEGA example use-case using standardized MPT benchmarking datasets mimicking viral particle movement in infected cells. Time series image from the Chenouard et al. MPT benchmarking dataset (Chenouard *et al*, 2014) corresponding to scenario IV, SNR = 7 and low particle density, was subjected to MPT within OMEGA. As expected most resulting trajectories displayed a behavior mimicking Levy flights (Levy, 1937), such as what observed in active motion. While most trajectories appeared to be valid, trajectory nr. 424 appeared to be the result of two erroneously linked particles, which was also confirmed by the appearance of a clear bend in the MSS curve (panel C, bottom left, *arrowhead*). When trajectory nr. 424 was edited using the Trajectory Editing plugin (panels A and D), the resulting allowed to split this trajectory in two individual trajectories 424.1 and 424.2 (panels E and F), one of which (nr. 424.1) gave rise to a straight MSS curve, consistent with the behavior of a uniformly mobile particle (panel F). After editing, trajectories were subjected to diffusivity analysis (panels G-K), yielding trajectories clustering in the top quadrant of the phase space graph (panel J, *red circled area*) as well as the prevalence of SMSS values close to 1 (panel K).

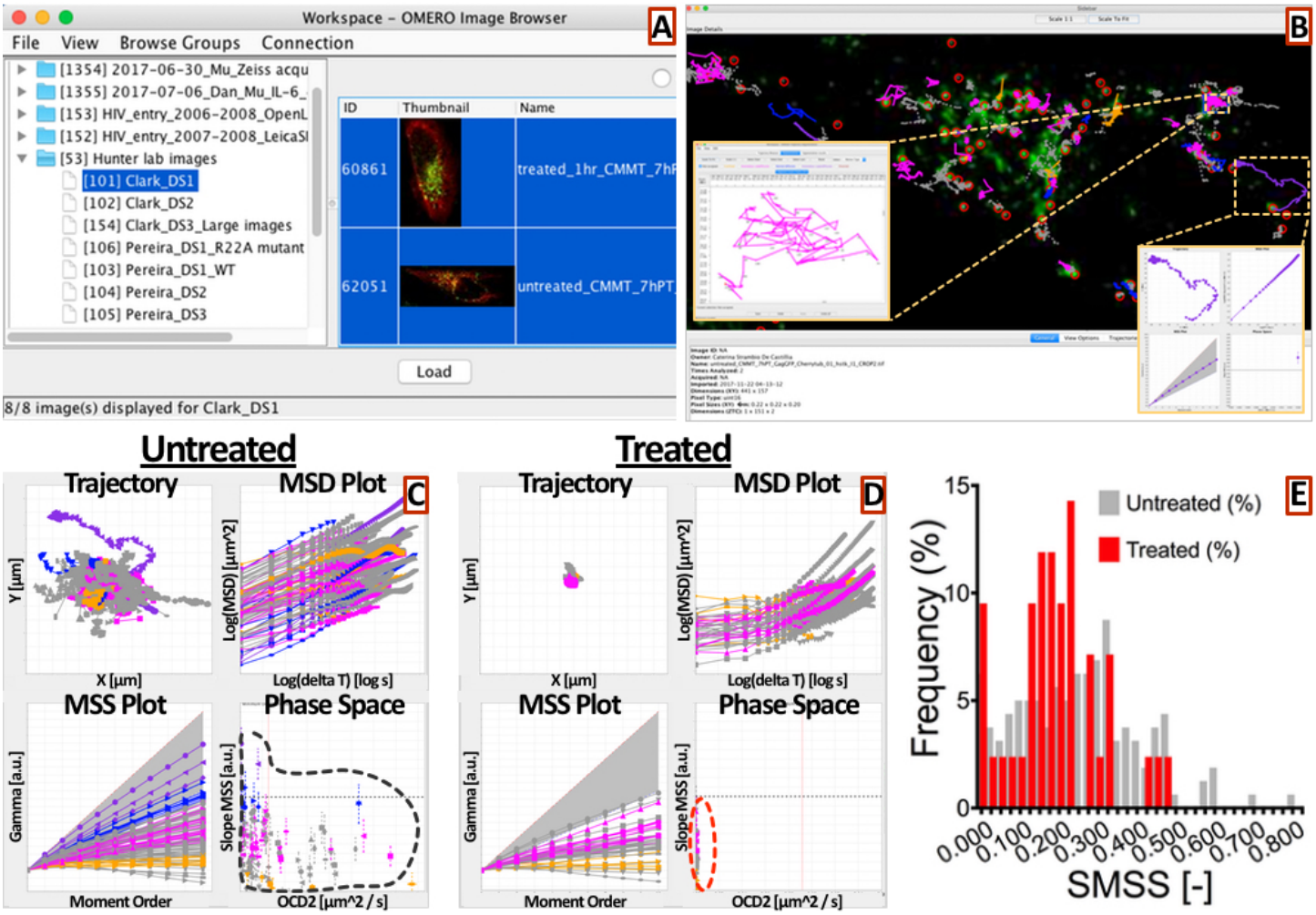

Figure 8

OMEGA example use-case using real-life imaging data: treatment with Nocodazole drastically reduces Gag-containing viral particles during M-PMV viral assembly. M-PMV producing, rhesus macaques CMMT cells were co-transfected with pSARM-GagGFP-M100A and p-mCherry-Tubulin to visualize the cytoplasmic assembly and trafficking of immature viral particles (Clark *et al*, 2013; Pereira *et al*, 2012; Pereira *et al*, 2014). 7 hr post-transfection cells were either mock-treated (*Untreated*) or treated with Nocodazole for 1 hour (*Treated*), before microscopic observation under 60X magnification using a Delta vision deconvolution fluorescence microscope (Applied Precision Inc., Issaquah, WA), equipped with a Cool Snap CCD camera. All acquisitions were performed at 37°C in a micro chamber with CO2 infusion. 3D images (with 10 z-focal sections spaced 200 nm apart) were in captured every 5 seconds for a total of 2 minutes. Images presented here are maximum projections of all z-sections in one plane. (A) After acquisition images were loaded onto OMERO and imported into OMEGA using the OMEGA image browser. (B) Images were subjected to single particle tracking using the OMEGA implementations of the MOSAIC particle detector and linker (Sbalzarini & Koumoutsakos, 2005). The resulting particles and trajectories were overlaid over the corresponding image using the OMEGA side bar image viewer. All trajectories were subjected to diffusivity analysis using the OMEGA DTM plugin. Trajectories that displayed a straight MSS plot curve were assigned the corresponding motion type using the OMEGA Trajectory Segmentation (TS) plugin. Insets display the motion-type assignment graphical user interface for a representative sub-diffusive (*fuchsia*) viral particle and the motion type classification 4-plots set (xy, MSD vs. t log-log, MSS and D vs. SMSS phase space plots) for a representative super-diffusive (*purple*) trajectory. (C) Global view of all identified trajectories for a representative image obtained from Untreated cell using the motion type classification 4-plots set. (D) Global view of all identified trajectories for a representative image obtained from Nocodazole treated cells, using the motion type classification 4-Plots set. (E) Comparison of the SMSS values frequency (i.e. relative frequencies expressed as percentages) distribution obtained with Untreated vs. Treated cells.