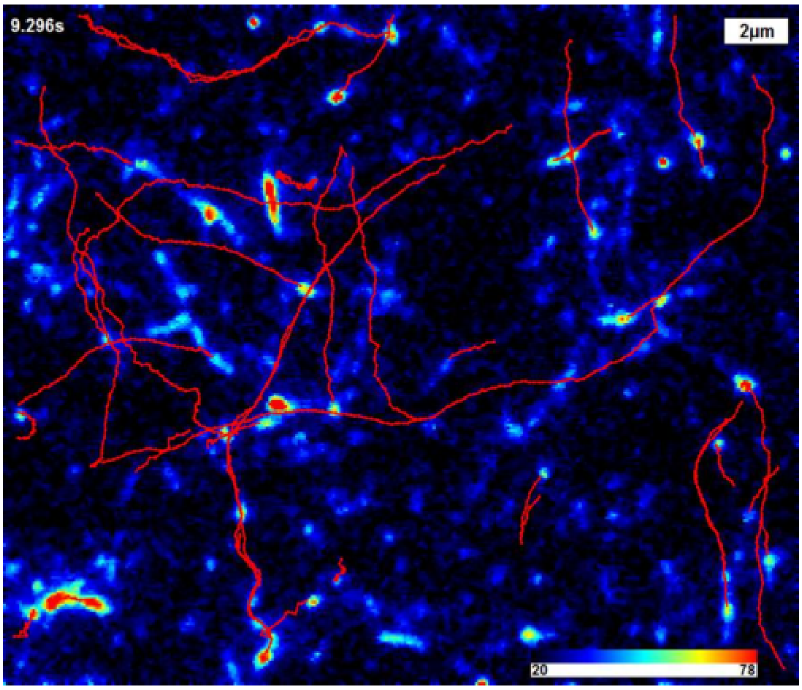
GMimPro



www.nimr.mrc.ac.uk/GMimPro

Table of Contents

Overview

"GMimPro" is an image sequence processor written by Gregory Mashanov (MRC, National Institute for Medical Research, London). The software was designed for detection and tracking of individual molecules observed by fluorescence microscopy. However, it may also prove useful for other image sequence analysis applications. Output data is stored in a text format suitable for further analysis and display using Excel spreadsheets or statistical packages.

The input data format consists of a sequence of digital images referred to here as a "Record". Individual images comprising a record are referred to as "Frames". It is assumed that in most cases image frames are acquired at roughly equal time intervals throughout the record.

GMimPro reads digital data records that have been stored using a proprietary format with a file extension ".GMV". This format is described explicitly in this document and it is straightforward to convert data files to or from this format to other commonly used formats. GMV files consist of a series of individual frames each of which is preceded by header information that contains information about: Time at which the frame was acquired (in milliseconds since the beginning of a record); X and Y scales (in nm/pixel); Frame size (in pixels); Top-Left corner coordinates of the frame used to define the start location if a region of interest (ROI) was saved during image acquisition (now a common feature of many digital cameras). Note: that Left and Top coordinates in children records reflect the position of the frame relative to the absolute camera Top-Left corner. Other, more system-specific information is also stored in the header (see GMV header description).

The dynamic range of each pixel can be either 8 or 16-bits/pixel (i.e. 1 or 2 bytes) and this can vary between individual frames that comprise a single record. For instance, if all the pixels on a frame have values < 256 counts (8 bits), then that frame is stored in 1 byte/pixel format. Otherwise the frame is stored as 2 bytes/pixel format. This method is used to reduce the overall file size.

GMV files are generated by image acquisition software that we have written for a variety of digital and analogue cameras (GMandor, GMpicolo, GMvideoUSB, these image acquisition programmes are available on request). In most cases it will be best to import data as a sequence of Windows® bitmap files (**8-bit grey scale** ".BMP" files) or as a sequence of "raw data" files (see ImageJ website at http://rsb.info.nih.gov/ij/features.html). These file formats contain raw pixel intensity data that can be imported to GMimPro (See Import Data). Other commonly used file types (like TIFF) will need to be converted into raw data file format using "ImageJ" or other software.

The main purpose of the GMimPro software is to automate detection and tracking of individual fluorophores. The theoretical basis of the two main image processing algorithms: Single Fluorophore Detection Algorithm (SFDA) and Automatic Single Particle Tracking (ASPT) is described in: ***G.I. Mashanov and J.E. Molloy (2007) "Automatic detection of single fluorophores in live cells", Biophysical Journal V.92, N. 6.***

A detailed description of each of the main modules and functions is given in the following sections of this help file. The names of menu items are given in **Bold** whilst Shortcut key combinations are shown in [square brackets]. Output data will be saved in tab delimited text format (.txt). If you need more information, or you find errors or bugs please contact:

Dr. G. Mashanov
Division of Physical Biochemistry
MRC National Institute for Medical Research
The Ridgeway, Mill Hill
London, NW7 1AA, UK
email: gmashan@nimr.mrc.ac.uk

Tel: +44 (0) 208 816 2110
FAX: +44 (0) 208 906 4419

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GMV Header

int leftX,width,topY,height,exp_in_ms,fr_size,fr_time; // 28 bytes

// left-top pixel coordinates + image height, width in pixels

// exposure time in milliseconds, fr_size - in pixels, fr_time– time when frame

// was captured (counted from the beginning of the record (in milliseconds)

float X_nm_pix,Y_nm_pix; (scales) // 8 bytes (scales)

byte Igain,Vgain,bit_pix,bin; // 4 bytes (intensifier and camera gain)

// bit_pixel – how many bits per pixel (if > 8 than two bytes per pixel are used

// for frame encoding), bin– binning (for information only)

byte params;// bit0 - Laser 1 ON

// bit1 - Laser 2 ON

// bit2 - Laser 3 ON

// bit3 - Reserved

// bit4 - Median filter ON/OFF (1=ON)

// bit5 - Reserved

// bit6 - Gated illumination (part of Frame (1=ON))

// bit7 - NEW FORMAT (1=NEW)

byte addInfo; (currently set to 0, allows to expand header in future)

WORD las_power,temperature,illumTime; // 6 bytes (illumTime in ms)

48 bytes all together

Note: If you wish, you can read GMV format files directly into ImageJ using the "Import, RAW" option, accessed from the ImageJ "File" menu. You will need to know the x, y pixel dimensions and number of frames (which can be read from the bottom of the GMimPro screen) and you must specify the offset to the first image and gap between images as 48 bytes. Finally, you must ensure that all frames in the record have been saved as 8 either 16 bit format. Please see later notes on how to create GMV files from other image file formats.

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ROI

Region of Interest (ROI) – You can select ROI by moving the mouse cursor to the left-top corner of a desired area and pressing left mouse button. Then drag the mouse to right-low corner of future ROI and release the button. ROI will be marked by rectangle. Information about ROI coordinates and statistics will appear in second status bar at the bottom of main window. You can move ROI using [arrow] keys or change its sizes using [Ctrl] + [Arrow] keys combination. ROI can be very small up to one pixel. If you use ROI of certain size (height > 12 pixels, and width > 12 pixels, and total number of pixels less than 10000) the precise coordinates of single objects within the region will be calculated presuming that only one particle is present in the ROI at the time or one particle is much brighter then the others. The X-Y coordinates and *Q* value of the detected object will be displayed in the right part of a second status bar. Use Play Record [Ctrl + M] to make the measurements and display the results in M&S window.

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M&S

Measurement and Statistics (M&S)– This is a multifunctional window where results of measurements and calculations are displayed. If you select ROI on an image and play the record [Ctrl +M] average intensity of the pixels in ROI versus time will be shown in M&S window. You can use Mask to do complex measurements in ROI. If ROI is small enough and placed on image so that the distance to every image boarder from ROI is bigger than ROIs width and height then relative intensity of pixels in ROI (local background subtracted) will be also displayed. If ROI X and Y sizes are bigger than 12 and there are less than 10000 pixels in it then single particle tracking will be done on every frame played (as described earlier). The precision of tracking will depend on signal intensity, noise level, and Fitting Limit. To display XY tracking results check **Show XY coor** in **Show** menu of M&S window. If no object was found in the ROI or if there few similar objects moving in ROI at the same time the random numbers will appear on the status bar and on XY graphs in M&S window. You can change graphs properties [Ctrl + Q] in M&S window and also save the graphs as TXT or BMP or print it (**File** menu). You can use **Filters** and other **Settings** to process data in M&S window. The same applies then you will display individual tracks in SFDA or ASPT modules or other statistics like MSD versus dT plot. The rate of fluorescence decay in ROI (photobleaching) can be measured by clicking **Fit Bleaching** in M&S context menu (right mouse button). This can take some time to do fitting. The results will be displayed at the bottom of the graphs. Make sure that you selected **Show Intensity** in **Show** menu before you do fitting. When you move cursor along M&S window current graph X and Y values will be displayed in widow status bar. You can "drag" the graphs across M&S window holding left mouse button.

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Profile

Profile – This window will appear automatically when you plot a profile on the image. Place cursor at the point you want to start your profile, press right button, and drag the cursor to the finish point then release the button. The profile will be updated when you move profile (use [arrow] keys) or go to another frame. The window and profile on the screen will disappear if you click on image again or close profile window. Make sure that you use appropriate Profile Width which you can set in main window **Settings**. The pixels across profile will be averaged. Do not allow profile corners overhang the edges of image then you use wide profile – software does not check the limits and the results of measuring will not be correct. You can choose the units for X axis in a context menu (right mouse button). It can be in micrometers or in pixels. You also can change graph settings or save the measurements using context menu. When you move cursor along profile window current X and Y values will be displayed in window status bar.

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File Menu

Open File

Open File [Ctrl + O] – This will open image records that conform to the ".GMV" format. Note: you can open only one file (record) at a time. If desired you can of course run multiple copies of GMimPro to deal with more that one record simultaneously. However, since this does not offer benefits in terms of processing speed it should probably be avoided. Please see GMV Header description and Import Data if you wish to convert files to or from GMV format.

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Save Current Frame

Save Current Frame – Enables the user to save the current frame or selected ROI as a Windows® bitmap file (24 bit per pixel, using the current colour palette) or as a GMV file containing just one frame [Ctrl + S]. Please note that it is difficult to refresh an image on the screen if the GMV record consists of just a single frame. Saving a single, averaged frame can be useful for background subtraction or for printing or documentation purposes.

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Play Record

Play Record [Ctrl + M] - This option will play a record on a rapid frame-by-frame basis, i.e. like a video display. If you have selected an ROI and then play the record back the results of various calculations will appear in a separate Measurements and Statistics window at the end of record. Use the [Esc] key to abort playback. ASPT and SFDA modules use this same function to do calculations and sometimes the record will be played automatically when the analysis proceeds. For information: the software loads only one frame from the record at a time but the operating system (i.e. Windows®) buffers as many frames as it can into the computer RAM – basically more RAM more speed – we find that computers with 1 or 2 Gbytes of RAM work well.

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Fuse Records

Fuse Records - You can concatenate several records into a single, contiguous record using this function. The time stamp used in the header information will increase from one section to the next using the original timing information (i.e. the timestamp value of the final frame of the first record saved will be added to all of the frames of next record and so on). By default, a blank frame will be inserted between original records to mark this point, but you can "uncheck" this option in **Settings** menu. In order to **Fuse Records** choose the option and select the required group of files in the "Open File" dialog window (all files should be in the same folder). Use [Ctrl] or [Shift] keys to make a selection. Then click "Open" and enter a new filename in the "Save File" dialog. The software will read in the selected files one-by-one in order and then save them to new, single GMV file. This might take some time if the original files are very large. If the file order is "jumbled" or for some reason not in the order that you require, then you can simply alter the original filenames so that they are listed in the correct order (i.e. by appending a, b, c, d, e.. etc to the start of each filename). However, the chances are that they will be in right order to start with!

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Print Image

Print Image [Ctrl + P] – this option will send the currently displayed image to a printer. The image will be printed exactly as displayed. If you wish to use this facility to quickly print images in order to document your work (i.e. to paste into a lab notebook) you might consider inverting the greyscale – the output will often look better and it saves ink! Also, see Print Zoom for printing adjustment.

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Next Frame / Previous Frame

Next Frame [Space] and **Previous Frame [BackSpace]** allow you to step through the record one frame at a time. You can also use the track bar at the bottom of the main window to navigate through the record. You can skip forward and back through the record by holding down either the [Space] or [BackSpace] keys.

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First Frame

First Frame [Home] – Rewinds record to the first frame.

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Batch Analysis

Batch Analysis - This option is used to do automatic single fluorophore analysis (ASPT and SFDA) of several GMV files without the need of operator intervention. The results of analysis will be saved as a succession of output data files which can then be read back into GMimPro to inspect that the analysis of ach file has run correctly. You need to specify some starting parameters for the ASPT and SFDA algorithms before initiating batch analysis. To do this, select the type of analysis you want (**Single Particle Tacking** in ASPT module or **S-test**, **P-test** in SFDA module). In the "Open File" dialog window select group of files for analysis (use [Ctrl] or [Shift] keys) and click "Open". The files will be analysed one by one and the results will be saved as GMI or TXT files (SFDA results). The name of the file will contain the name of the original GMV file + details of the parameters used for analysis. This option is useful if you need to analyse a large number of files on a slow computer.

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Import Data

Import Data - This module is used to convert RAW and BMP files into GMV files. You can use "ImageJ" or other software first if you need to convert your starting image sequence into a RAW image format. You will then use the **Import Data** option and select the type of data to import, the X and Y dimensions of each frame, X and Y scales (in nanometres per pixel) and time interval between frames. You need to check the '**2 bytes per pixel"** check box if you wish to convert to 16-bit (2-byte) RAW data file. Then press '**GO**" to start conversion. You then select the RAW data file you wish to convert via the "Open File" dialog (and click "Open"). Then enter the name of the GMV output file using the "Save File" dialog (and click "Save"). The number in the lower left corner will show the number of frames converted. Similar actions should be taken in order to convert a series of RAW data files. Ensure that files are numbered sequentially at the end of each filename (before the extension e.g. test1.raw, test2.raw, test3.raw…. etc.) and simply select the first file in the sequence and press "Open". GMV files will be generated with matching index numbers (Note: there is a 10000 frame limit for any one file). The same procedure allows conversion of a series of BMP files (8-bit per pixel, grey scale). If you have difficulty converting a series of many frames into a single GMV file directly. Then you can try using "ImageJ" to convert your series of individual RAW or BMP frames into a single, concatenated, RAW data record (composed of many frames) and then work with that (as described above).

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Save Record As

Every Frame as:

A “decimated” record in which a reduced number of frames are created that are the sum of a number of specified frames calculated from the original data record. For example if the original record has 100 frames and you select **One Frame as Sum** of **5 Frames** the newly saved record will contain 20 frames. Summing the pixel intensities in this way mimics longer image exposure times (i.e. image integration). If you have an analogue camera system running at standard video rate 25 or 30 frames/sec you may wish to integrate images using this technique and increase the effective exposure period for each stored frame (e.g. reducing the effective frame rate to 2.5 frames/sec if you resave the data as the sum of 10 frames). The summing procedure works better than averaging as it avoids rounding errors (particularly for 8-bit raw data sets). The effective exposure time will be stored in the GMV header according to the summation value.

You can also choose to save data as a 5 frame median filter **Median of 5.** In this case every frame in the output record will contain the median value of pixels calculated from 5 frames in the original record. Again, the output record will contain 5 times fewer frames than the original record.

Record decimation using either of the above two methods is a convenient way to reduce the overall file size of the original files. Time resolution is sacrificed but we have found image intensity integration, or median filtering can prove beneficial for some data sets.

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Part of the Record

Part of the Record – Enables you to save part of a record (or its ROI) as a new record (You need to select the ROI first if you want to save ROI only). You will need to input the frame numbers of the first and last frames to be saved, using the dialog window and specify a new filename in Save File dialog window.

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Save Record with Binning

Save Record with Binning - You can reduce the spatial dimensions of a record by binning pixels. Every pixel in an output record will then contain the sum of 4, 9 or 25 pixels (2X2, 3X3, 5X5 binning). X and Y scales stored in GMV header will be increased according to binning values. Note that the whole frame will be binned and saved even if ROI was selected.

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Record with threshold subtraction

Record with threshold subtraction - This option is useful when pixels in a record contain a constant intensity offset. For example: we find that some EMCCD cameras have an offset of 900-1000 counts and then the signal change of interest might be only 100-300 counts on top of this value. This can make image display awkward. Removing the offset might well significantly reduce the size of final data file. To perform this procedure you must first set the threshold value using the LUT window (see later notes). To do this, open the LUT window and use grey level controls [**+]**, [**-]** keys or [I], [D] keys to change threshold (red vertical line). The value will be displayed at the top of the LUT window. All pixels with values ≤ threshold will be black on the current image. Then go to **File**, **Save Record as:**, **Save Rec. with Thresh. Subtr**. Choose a name for your new record. Then all pixels in the new record that you create will have values reduced by the threshold value. If some pixels in the original record had values that were actually less than the threshold then their values will also be set to zero (and not negative values).

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Record with Inverse Intensity

Record with Inverse Intensity - This is necessary in order to track dark objects on a bright background and might be useful for other purposes (e.g. saves ink when printing files for data documentation). This procedure will save a new record in which intensity of each pixel is inverted. The maximal value used for inversion is determined by the maximal number of bits used to encode the brightest pixel in that particular frame. For example: if this requires 11 bits, new pixel values will be calculated as 2048 minus the original pixel value.

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Thin out Record

Thin out Record – This is another form of decimation that is useful if image multiplexing has been performed (i.e. alternate frames might use different filter sets or have been collected at different levels of defocus, or TIRF angle etc.). Such records then need to be split into new data files that contain the separate information. Using the "Thin out Record" option you can save one of N frames in the original record starting with a particular offset value (e.g. from frame N=2, save every third frame from the original record). Note that frame numbering, in the GMV format, starts from zero. You can also use this option simply to reduce the size of the data records. However, the "**Save Record As"** option (described earlier) would be a more elegant way to achieve this.

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Save as RAW Data File

Save as RAW Data File - Data from a GMV file can be saved as a RAW data file. If any pixel in a record has a value more than 255 counts then all data in the RAW file will be saved in 2 bytes/pixel format (16-bit, Little-Endian byte order), otherwise it will be saved in 1 byte/pixel format.

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Save as Sequence of RAW Files

Save as Sequence of RAW Files - Every frame in a record will be saved as a separate RAW data file. If any pixel in the original record has a value > 255 counts then all data will be saved in 2 bytes/pixel format (16-bit, Little-Endian byte order), otherwise it will be saved in 1 byte/pixel format. Note: that the whole frame will be saved, even if an ROI was selected. You can save an ROI in a separate GMV file if you want to then convert it to separate RAW data files. You will need to specify the new filename in the "Save File" dialog window. The filename must not have any extension or number at the end. An ascending index number will be generated and appended to every RAW filename so that the sequence order is known. This form of filename indexing is consistent with many other image processing packages (including ImageJ).

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Save as Sequence of BMP Files

Save as Sequence of BMP Files - Every frame or its ROI will be saved as a separate Windows® bitmap data file (24 bit/pixel, using the current colour palette). This option is used to generate a series of BMP files that can then be used to create a single movie-format file (e.g. AVI, MPEG, MOV) for presentations. There are several commercial packages for this purpose, e.g. the inexpensive “VideoMach” software (see e.g.:http://www.gromada.com/videomach.html). You will need to choose an appropriate palette and adjust contrast and brightness (using the GMimPro LUT settings) and also add Scale Bar, Time mark, and other items before you save the sequence. When specifying the base filename you must not have a file extension or use a number as the last character. An ascending index number will be appended automatically to every BMP file generated.

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Save as Sequence of BMP Files on Page

Save as Sequence of BMP Files on Page - Every frame or its ROI can be saved as a string of images (10 images in a raw) in a single Windows® bitmap data file (24 bit/pixel).

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Settings Menu

Full Width at Half Maximum (FWHM)

Full Width at Half Maximum (FWHM) - this parameter determines the size of single particles (in nanometres) which will be tracked using the ASPT module or in the ROI analysis. For single molecule tracking this parameter should be the point spread function of the imaging system – which should be close to the diffraction limit (e.g. for 500 nm green light FWHM~250 nm). However, if the software is used for other purposes the value can be adjusted to suit – you will need to experiment to see what works best. After you enter a new value, a spot will be displayed at top-left of the image to indicate what the target object looks like.

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Fitting Limit

Fitting Limit - GMimPro uses a Gaussian fitting method to find the precise (floating point) XY coordinates of a single particle. If the image of a particle is sufficiently bright and the noise level low the XY coordinates will be determined with a precision much better than the size of a single pixel (in fact down to a fraction of nanometre). The **Fitting Limit** determines the limit at which fitting iterations stop. It is measured in fractions of a pixel. If we set it as 0.5 pixel it will measure the object position to 1/4 pixel size because fitting precision increases as 1/(2n), where n increases from 1 to 11. If we set Fitting Step to 0.01, software will track objects with precision of 0.00781 of pixel size. To find the position of many objects with high precision requires a huge number of calculations. So, it is wise to set an appropriate fitting resolution – knowing the likely signal to noise of your images – otherwise you just waste computing time and fool yourself about the final accuracy of the fit! You can calculate the expected precision – or you can estimate the level of positional noise by direct empirically by imaging immobile control specimens under identical conditions – setting the fitting limit as small as possible (so that you get the best possible fit and any noise is then "real noise" and not noise generated by poor fitting). Then measure the apparent XY fluctuations of the tracked objects (RMS) using ROI and M&S window. You then know a useful value for the **Fitting Limit** (obtained by dividing the RMS positional noise by the size of a single pixel).

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Print Zoom

Print Zoom - Print Zoom can be adjusted to produce the desired size of a printed image or graph.

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Velocity Step

Velocity Step - This parameter is used in the ASPT module to give an average value of the velocity of tracked objects. If Velocity Step is > 1 then velocity is calculated over that running interval. For example: if Velocity Step = 5, velocity is calculated using object coordinates on frames 0 and 4, 1 and 5, 2 and 6, and so on. It is particularly useful to measure the velocity of slowly moving objects when the noise level is high.

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Scale Bar Size

Scale Bar Size – Set the size of scale bar in μm. Check Show Scale Bar line in View menu to put scale bar on image.

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Profile Width

Profile Width - You can use right mouse button to draw a profile (of variable width) on the image. The pixel intensities along the long axis of the profile will be shown in a Profile Window. Pixel values are the average across the profile width. Set Profile Width to 1 if you want to see the intensities of individual pixels along the line.

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Sum Buffer Size

Sum Buffer Size - This parameter is used in Running Sum Filter. The buffer size determines how many frames are used to calculate the running sum (from 2 to 10 frames). The bigger the buffer value the greater the period of integration - but fast dynamic changes will be smoothed.

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Add Blank Frames

Add Blank Frames - option is used in Fuse Records function. It separates individual records in output file with two blank frames. This prevents the linking of independentent tracks in "fused" records.

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Local Backgr Rad

Local Backgr Rad - Set the size (radius) of the area used for Local Backgr Subtraction filter.

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Edit

Average Record

Average Record [Ctrl + A] – This function calculates the average value for every pixel on the image over the entire record and displays it as a single frame and this can be saved if you wish (see Save Current Frame function).

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Accumulate

Accumulate [F4] - This function adds the current frame to a special buffer (accumulator) and displays the resulting, averaged frame, and then moves automatically to the next frame in the record. You need to clear accumulator [Delete] to start accumulating again. Pressing F4 several times in succession will build up a locally averaged image.

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Make SD image of a record

Make SD image of a record [Ctrl + D] – This function calculates changes in pixel intensity (variation) during the record and displays it as a Standard Deviation of pixel intensity. This is useful when you need to find which areas on the image change most during the record (these will be represented as bight pixels) and those which stayed relatively static (dim pixels). You can save the result using Save Current Frame function.

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Inverse Intensity

Inverse Intensity [Ctrl + I] – This function finds brightest pixel (maxValue) on current frame and inverts the intensity of all pixels (maxVal-pixelValue) on current frame.

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Rotate Frame Clockwise

Rotate Frame Clockwise [Ctrl + R] – This function rotates the current frame clockwise. It can be useful for presentations.

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Clear Accumulator

Accumulator [Delete] – Clears accumulator. (See Accumulate).

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Subtract Offset (LUT threshold)

Subtract Offset (LUT threshold) – This function subtracts an offset value from every pixel on current frame. The offset threshold value is set using the LUT window. This function can be used to create a Mask which can be used in GMimPro or to check the effect of threshold subtraction which will be used during the save Record with Threshold Subtraction function.

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Enlarge Image

Enlarge Image – this function stretches the current frame 2 times in X and Y directions (4 times i.e. more pixels). It zooms the image by creating a smooth interpolation between original neighbouring pixels.

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Convert to Percent Image

Convert to Percent Image – Scales every pixel on the current frame from 0 to 100 relative to the brightest pixel on the image.

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Find Single Particles

Find Single Particles – This function finds objects matching Single Particle criterion, set using the FWHM value (Settings menu). The result of the procedure is an image where bright pixels mark the position of objects matching the shape of putative single particles. Equation 8 from *Mashanov and Molloy* paper was used to calculate pixel values (indexes $Q(x,y)$) on the resulting image. The $Q(x,y)$ values are multiplied by 10 to give a smooth representation of Q values that otherwise fall in the range of 2.0-5.0 which is not suited to pixel encoding and display. So, remember that pixel intensities read from the screen should be divided by 10 to get the Q values of *Mashanov and Molloy* (See ASPT module for future use of Q values in Q-Threshold). An, 11 by 11, pixel mask or kernel is used in these calculations. Therefore it is desirable that the FWHM of detected objects is close to this mask size / 3 or 3 pixels. If the FWHM of the viewed object is 250 nm, the optimal pixel size, in terms of the viewed object dimension, should be 80-90 nm.

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View

Look Up Table (LUT)

Look Up Table (LUT) – this window opens automatically by turning the computer mouse wheel. This window shows the distribution of pixel intensities on current frame and a graph showing the applied LUT transfer function - representing correspondence of pixel intensity in the data file and pixel intensity represented on the screen. The LUT transfer function can be made steeper or flatter (contrast) or shifted left or right (brightness). Mouse wheel, mouse buttons or arrow keys will change the LUT. You can also use non-linear Gamma function or Sigmoid LUTs. However, if your camera response is linear with intensity (many camera manufacturers strive to achieve this) then colour on the screen will no longer be proportional to original intensity. The LUT window also shows the current palette used for painting and the intensity threshold (default threshold value is zero). You can change threshold using Grey [+] and [-] keys on numerical keyboard or [I] and [D] keys on main one. All pixels below threshold will have black colour on the screen but threshold itself doesn't affect pixel values. LUT and palette also do not affect the original data– just the way it is displayed on the screen. You can **Reset LUT** to linear 1:1. The Main-LUT menu option will appear if you have loaded a Ghost image and need to adjust its LUT separately.

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Palette

Palette - Images can be represented in different palettes. Simple palettes are Grey and Green. The pixel intensities are gradations of grey or green colours (256 colours from 0 to 255). The colour – intensity correspondence can be checked in the LUT window. The palette bar can be placed on the image for reference (See Show Palette). There are also two multicolour palettes RYW (Red-Yellow-White) and BYR (Blue-Yellow-Red). The simple Red palette is reserved for Ghost image. We recommend using the Green palette if you put a Ghost image on screen. Then the overlapping bright pixels of main and Ghost image will be yellow.

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Zoom In and Zoom Out

Zoom In [Ctrl + Z] and **Zoom Out [Ctrl + X]** used to change the zoom on the screen. If the zoom is 3 then every pixel on the frame will be represented by 9 pixels on the screen and so on. Current zoom value displayed in the main menu (e.g. View(Z=3)).

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Show Time

Show Time – The actual time the frame was captured will be shown on the image. You can drag the time label to the desired location on the image using the left mouse button.

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Show Scale Bar

Show Scale Bar – check this option if you want to display a scale bar on the image. The size of scale bar is set in Scale Bar Size menu in Settings menu. The length of the bar on the image is determined in the frame header (see GMV_header description) "size of scale bar" / "scale (nm/pixel)". You can drag the scale bar to the desired location on the image using the left mouse button.

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Show Grid

Show Grid – The grid will appear on the image for measurements or for presentations. The cell size of the grid is the same as the Scale Bar Size.

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Show Palette

Show Palette – A bar showing the current Palette will appear on the image showing the intensity of the darkest and brightest pixels in the corresponding LUT. Note: if a nonlinear LUT was used the change in colours will not vary linearly with intensity. You can drag the palette bar to the desired location on the image using the left mouse button.

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Show File Info

Show File Info – a string showing the path to, and the name of, the current file will be shown on the top of image.

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Show Outline

Show Outline – It is possible to draw a free hand line on the image by pressing the mouse wheel or middle mouse button and moving the mouse cursor. The line will contain a chain of dots. This outline can be used in the ASPT module or for presentation. You can Clear Accumulator [Delete] to start drawing again.

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3-D Image

3-D Image – You can create a 3-D representation of the intensity data of the current frame or of a selected ROI. In the 3-D window you can adjust settings such as: Background (black or white), Z-scale, Image offset from the top of the window (**Vertical Offset**), **Image Tilt** (relative to viewer). Note: if LUT, Palette, and Zoom are changed in a main window this will also affect the 3-D image but will not be seen during the changes. You need to **Update [Ctrl + U]** the 3-D image to see the changes. You also need to **Update** if you move to another frame in the record or do any other operations in other modules. This is because building the 3-D image requires a large number of calculations so redrawing is relatively slow. In the **Show** menu, you can choose to show 3-D image **as Blocks** (Palette will not affect this mode) or put a **Grid** separating individual pixels. If you choose to Show Time or Show Scale Bar or Show Palette in a main window it will also appear on a 3-D image. Press middle mouse button or mouse wheel to put 3-D scale bar on desired position on 3-D image.

You can print a 3-D image or save it as 24-bit BMP file. You can also save the whole record as a sequence of BMP files, which you can later convert into a movie (Use **Save as Sequence of BMPs** in a **File** menu of 3-D window).

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Mask

Mask

Mask – You can load a single image which will work as a "mask" required for some operations and measurements. The image should have been stored in a GMV file and should have the same dimensions (x,y) as the loaded image sequence (otherwise no mask operations will be done and no warning will be given). Such a file can contain a number of frames but only the first one will be used as mask. You can load the same record you opened in GMimPro if you want to use its first frame as a mask. You may wish to specially prepare the image you want to use as a mask. For example: You can use the Subtract Offset to threshold an image and use Save Current Frame to store the frame as a mask. Then use **Load New Mask** and check **Boolean Mask** in **Mask** menu. Now pixel values throughout the record will be set to zero wherever mask=0. Pixels with zero intensity will not be counted in ROI measurements (M&S window) if the **Boolean Mask** option is checked. If you have uneven background intensity you can create a separate experimental record or average and smooth an existing record to create a background mask – then use **Subtract Mask** in the **Mask** menu. If subtraction produces negative values they will be zeroed. You can use only one option (**Subtract Mask** or **Boolean Mask**) at a time. Mask operations are done before filtering operations. You can **Unload Mask** to remove the mask frame and its functionality.

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Temporal Filtering

Temporal Filters (overview)

Temporal Filters – These filters act upon individual pixels within a record sequence. The filter operates as a running box of variable duration. Note: Temporal filtering is be done after Spatial Filtering. Click on the filter name in the menu to activate or deactivate it.

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Median Filter (3, 5, 7 frames)

Median Filter (3, 5, 7 frames) – This running filter finds the median value of pixel intensity in 3-7 frames and puts this value in the current frame. Please note that the changes in pixel intensity will be delayed by 1-3 frames when you play the record because the software doesn't read the frames ahead of current frame. This filter is useful for noisy records where random (photon) noise affects pixels on neighbouring frames. This filter will start to work after it collects a specified number of frames after beginning of record.

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Subtract Previous Frame

Subtract Previous Frame – this filter subtracts the previous frame from the current one. If subtraction produces negative values they will be zeroed. This filter can be used to detect moving objects.

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Running Sum Filter

Running Sum Filter – This filter sets the current frame to be the sum of a specified number of previous frames. Set the number of frames you want to sum in **Settings** (Sum Buffer Size). This filter is useful when you have a weak and slowly changing signal and cannot increase exposure time or illumination intensity to improve it. This filter will smooth fast changes but will increase the amplitude of a signal. This filter is better than using a running average because it avoids integer rounding errors.

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Spatial Filtering

Spatial Filters (overview)

Spatial Filters - These filters affect the pixel value according to values of neighbouring pixels on the same frame. Note: Spatial filtering is done before Temporal Filtering. Click on the filter name in the menu to activate or deactivate it.

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Spatial Median Filter

Spatial Median Filter – This filter finds the median pixel value in 9 or 25 pixels (3X3 and 5X5 filters) surrounding a given pixel and will replace it. This filter will reduce noise affecting individual pixels without significant smoothing of the image.

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Smoothing Filter

Smoothing Filter – This filter will calculate the average pixel value in 5X5 pixel area around given pixel and put it on current image. You can use it together with Sharpening Filter to create Super-Boost filter.

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Sharpening Filter

Sharpening Filter – This filter uses special kernel mask (5X5 pixels) to emphasize local details on image (first derivative). Each kernel mask element is multiplied by the corresponding pixel around the current pixel and the sum of all operations is then stored as the new pixel value. The value of the central pixel in a mask is 16. The value of 8 pixels surrounding it is set to zero and value of 16 pixels at the border of the mask is set -1. You can use it together with Smoothing Filter to create Super-Boost filter.

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Sobel Filter

Sobel Filter – This filter will emphasise edges on the image.

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Subtract Bkgr

Subtract Bkgr Filter – This filter is used to subtract the lowest pixel value (offset) in the local area around given pixel. The size of local area is determined by its radius in Settings menu (Local Backgr Rad). Please note that the area has square shape for simplicity. This filter can be used to subtract offset values on unevenly illuminated images.

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SD imaging

SD imaging

SD imaging – This procedure calculates the running Standard Deviation of a pixel's intensity over a specified number of frames. Pixels that show rapid intensity fluctuations will appear bright while non-fluctuating signals will be dim.

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Ghost Image

Ghost Image

Ghost – you can display a ghost image on the screen. It is useful if you want to compare two frames visually on the same image. You have to load a GMV format file as ghost file where its first frame will be used as ghost. The ghost frame should have the same X and Y sizes as the main record open in GMimPro, otherwise no ghost image will appear on screen and no warning will be given. The ghost image will be displayed using a Red palette so you are advised to use an appropriate Palette (Green) for the main images. You can use LUT to adjust main and ghost look-up tables separately pressing Main-LUT / Ghost-LUT in main menu in LUT window. Use [Insert] key to put on or remove ghost image from the screen. Ghost image doesn't affect any calculations.

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SFDA

Single Fluorophore Detection Algorithm

Single Fluorophore Detection Algorithm (SFDA) – This module is used to detect single fluorescent molecules immobilized on the substrate. In order for single fluorophores to be detected using SFDA they should appear/disappear rapidly during the record. The detailed description of the method is given in *Mashanov G.I., and J.E. Molloy (2007) "Automatic detection of single fluorophore in live cells", Biophysical Journal, V. 92, N. 6.* First, you must set the dimensions of an idealised single fluorophore (Spot Size) and the length of the time Window (the size of running average filter) which will be used for calculations. We recommend adjusting the magnification of the microscope/camera system so that FWHM of a single molecule occupies about 3X3 pixels. This means you can use 5X5 pixel spot size for calculations. We also recommend using fast enough imaging rate that the life-time of single molecules during record will spread along substantial number of frames which will give a number of data points so that a long window (up to 10 frames) can be used. Use Detect and Tracking to find single fluorophores in current record.

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Detect

Click **Detect** to find pixels that exhibit with sudden intensity changes. This procedure generates a so-called "drop" image in which pixel values are proportional to the largest intensity drop that occurred at that pixel location during the record. The value depends upon the window size and spot size used for detection. The procedure also creates a so-called "rise" image in which pixel values are proportional to the largest sudden rise in intensity during the record. The drop image is displayed as a pseudocolour image after the **Detect** routine is completed. Pixels with values less than a "Drop Threshold" will have zero values (Hint: use right mouse button to plot Profile and view pixel values). Adjust **Drop Threshold** using Up and Down Arrow keys. Changes are dynamic and the number of pixels above threshold will be displayed in the status bar at the bottom of the SFDA window. You can subtract the rise image from the drop image (**Action → Subtract Max Rise**). This procedure will remove pixels in which intensities both dropped and increased suddenly during the record. This can happen if a single fluorophore appears "lands" during a record (this is common with TIRF imaging methods). Alternatively, you can add the rise image to the drop image (**Action → Add Max Rise**) if you want to enhance the amplitude of pixels which both appeared and disappeared during the record. In that case, by increasing the drop threshold only newly appearing spots will be left on the image. To view the rise image choose **Show-Rise** line in the context menu (right mouse click on SFDA window). Local background is subtracted from the spot intensity by default. Check the option "**No Background Subtraction"** in the **Action** menu if you do not want to subtract background or you can use **Spot Size = 1x1** pixel.

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Tracking

Finally (after Detection is complete), press **Tracking** to store intensities of the pixels left on the image into memory for future grouping and selection. The number of pixels you can track is limited to 200000. (Hint: if this is a problem, split large images into 2 or more ROIs (Select ROI → Save Record As → Part of Record (from first to last frame)). The number of frames on which you can track pixels is limited to 1000. If you have longer records you can either "decimate" them or save them as the running Sum of frames (Save Record as → Sum of) or you can break the record into several sections (Part of Record) (see above).

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Analysis

Summary graphs will be displayed after tracking is complete. Graphs will show distribution of pixel intensities and distribution of life-times. Click right button mouse on a panel and choose **Set Graph** in a context menu if you want to change graph appearance. You can also change the intensity and time bins used to build the distributions (use context menu (right mouse button)). Press **Show Spots** in SFDA main menu to find pixels in the centre of local clusters. Statistics displayed at the bottom of the SFDA window then deal with those clusters not with pixels. The distribution of spot intensities (averaged according to the **Spot Size**) is fitted to a Gaussian function. Whilst the distribution of life-times is fitted to a single exponential function giving, τ, half-life of the spots (off-rate constant = $1/\tau$). The step-test or "**S-test"** (in **Drop** menu) selects spots which were present from the beginning of the record and disappeared in one step. The pedestal test or "**P-test"** (in **Drop** menu) selects spots which appeared some time after the start of the record and disappeared during the record (pedestal test). You can change S-test and P-test thresholds by pressing [Up] and [Down] arrow keys on keyboard (S-test and P-test have independent thresholds). The changes will be seen on the screen and number of spots above threshold will be displayed in the status bar at the bottom of SFDA window. The multiple-step test, **MS-test,** (in **Drop** menu) will select spots which were present on the starting image of the record and disappeared more gradually as a sequence of several separate bleaching steps (characteristic of multiple (i.e. up to 4 or 5) fluorophores). The **MS-test** is useful if you are dealing with immobile objects labelled with several fluorophores, for instance most commercial antibodies have multiple dyes attached.

You can navigate between individual spots by pressing [PgUp], [PgDn] keys on the keyboard. The intensity vs. time of each spot will be shown in M&S window together with a fitting function for S-test or P-test. The position of the current spot on the image will be marked by a blinking crosshair. You can move to the First Frame in a record [Home] or make a Standard Deviation image of a record to see all the spots on the screen. You can mark all the spots detected on the current frame by checking **Show Crosses** option in context menu (right mouse click on SFDA window), Note: **Show-Spots** menu should be clicked because the crosses will show the spot centres only.

You can save intensity and life-time distributions as BMP image files (Action → Save Graphs as BMP) or print it (Action → Print Graphs) or save **Distributions as TXT file** (see context menu). Go to context menu (right mouse button) if you want to change intensity and time bins in distributions. You also can save all the data in a text format file (**Action → Save Data as TXT**). The data will be saved as a number of strings (in "Tab delimited" format) containing information each detected object: drop time (s), drop amplitude (counts/pixel), rise time (s), rise amplitude (counts/pixel), background amplitude (counts/pixel), standard deviation of the signal when fluorophore was present, standard deviation of the signal when fluorophore was absent, value of S-test, value of P-test, X-position (in pixels), Y -position (in pixels). You can use Excel or other programs to perform further analysis or combine data from different files. Note: that S-test and P-test values will be saved without multiplication (by 10). You can also use our satellite software (SFDA-stat) to add results together and do some measurements.

You also can save raw data (**Action → Save X,Y, Intensity (TXT)**) in txt format. First row is time. In every row below: X and Y coordinates (in pixels) and intensity data points for every positively identified object for the length of the record. It is better to save information about spots not pixels because it will spare space and calculations. If you display spots intensity data will be averaged according to **Spot Size** option and **Background Subtraction** option in **Action** menu.

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ASPT

Automatic Single Particle Tracking (ASPT)

Automatic Single Particle Tracking (ASPT) – This module will track particles moving in the X-Y plane. The objects should have a 2-D Gaussian shape (which is true for single molecules) that is spread over a known number of pixels (see Find Single Particles procedure for details). The detailed description of the method is given in *Mashanov G.I., and J.E. Molloy (2007) "Automatic detection of single fluorophore in live cells", Biophysical Journal, V. 92, N. 6.* You must specify a number of parameters before you start ASPT using the **Settings** menu. Note: FWHM and Fitting Limit explained in other sections of this Help. Click Detect and then Tracking to to analyse current record.

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Detect

Click **Detect** to start detecting single particles with specified FWHM usingQ-Threshold. Software will find single particles on the current frame and link them to the nearest particles found within Max Radius on the previous frame. The number of detected objects on each frame and the number of objects for which tracks are generated (>Track Length) will be displayed in the status bar at the bottom of ASPT window. The left graph will show the distribution of intensities of the detected objects on the current frame and the right graph shows distribution of distances between closest objects on the image. You can press [Esc] to terminate Detection before the end of a record is reached but only this part of the record can be analysed later.

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Tracking

When detection is finished you can click **Tracking** to start gathering information about the precise X-Y location of detected objects. If Fitting Limit is set to a small value and the number of detected objects is high this process can take a significant time because it involves a substantial number of calculations. When tracking is complete the distribution of intensities of all tracked objects will be displayed on left panel and distribution of coefficients of lateral diffusion (*Dlat*) of individual objects will be displayed on right panel. Click right mouse button on a panel and choose **Set Graph** in a context menu if you want to change chosen graph appearance.

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Analysis

The Mean Squared Displacement (MSD) versus dT (time lag) graph will be displayed in Measurements and Statistics (M&S) window. If the data fits a straight line then the process is well described by a random walk or Brownian motion. Black line on graph will show the number of objects for which tracks were long enough to add information at this time lag point. Note: you can save MSD data using **File** menu in M&S window. You can get access to individual tracks by pressing [PgUp], [PgDn] keys. MSD versus dT dependence for individual track will be shown in the same M&S window. You can also see individual spot intensity (**Show** → **Show Intensity**) and X and Y coordinates (**Show** → **Show XY coor**) in M&S window. You can stretch the graphs in M&S window if you check **Stretch Graphs** in **Settings** menu. The track of the displayed object will be shown blinking on image. You can select a part of the track if you display XY coordinates in M&S window. Hold [Ctrl] key and hold left mouse button to select part of the track. Then click right mouse button and select "Cut Selected Part" in the context menu. Only selected part of the track will be used for future analysis. **NOTE**:It is not possible to undo this operation. You can mark an individual track as "GOOD" or "BAD" pressing [CR] key ("Enter") while your active window is M&S. Tracks marked as "BAD" will not contribute to distributions of other measurements and will not be saved in data files. You can see all tracks on the image (press [S] key) or remove them (press [S] key again). You also can display tracks in dynamic mode then only the tracks of the objects tracked on current frame will be displayed and their tracks will end on current frame. Press [T] to show it and press [T] again to remove it from image. Play record [Ctrl + M] with show track option [T] switched on to make sure that you satisfied with the quality of tracking. You can see pseudo-colour representation of the motilities (*Dlat*) of all detected tracks (**Show** → **Kdiff Tracks**). Pixel's intensity will represent (*Dlat*) of a track placed in this area in um2/s units multiplied by 1000 to get enough value for integer pixels. If few tracks overlap then pixel values will be averaged. You can also display the average distance the objects travelled from the point they were first detected (**Show** → **Dist. from Origin**). It should be a straight line if objects move more or less linearly with constant velocity. You can display the distribution of average object's velocities (**Show** → **Velocity Distrib.**). After displaying desired distribution you can save it in TXT format for future analysis and representation (**Data** → **Save Distrib. as TXT**). You can also save intensity and X-Y tracks of all detected objects (**Data** → **Save Int + XY Tracks (TXT)**). The intensity values will be saved in counts/pixel units and X-Y coordinates in micrometers.

Tracking results can be stored in a special format in GMI data files which contain information about time reference point and precise intensity and XY coordinates for every frame this object was present (detected) on image. After you have finished tracking you can save such a file (**Data** → **Save X-Y-Int Data (GMI)**) and read it (**Data** → **Read X-Y-Int Data (GMI)**) at a later date to avoid tracking numerous objects in big files again. You can analyse many files in batch mode (**File** → **Batch Analysis** → **Single Particle Tracking**) overnight and see the results next morning (use [Shift] or [Ctrl] keys in Open File dialogue window to select group of files). Some **Settings** used for analysis will be added to GMI filename which will have the same name as analysed GMV record. If you want to put together data from a few records you can Fuse Records and do ASPT analysis of a joined record or you can use satellite software (Motility) to do statistical analysis of big data sets. Note: Do not forget to check Add Black Frames option in **Settings** Menu (Main Window) if you are fusing records to avoid false connection of objects between records. You can link tracks which had one missing data point at a length ≥ Track Length using (**Data** → **Link Tracks**) after you finish "Tracking". The position of the object on the last frame before missing point should be within Max Radius of the object's position on the frame where spot reappeared. The linking will be done in Batch Mode of ASPT if you leave Link Tracks in Batch Mode option checked in **Settings (ASPT window)**.

You can draw a free hand line on the image if you check **Show Outline** in **View** menu. For example draw a line around a cell with moving molecules inside it. The line will be a chain of dots. You can choose **Dist. to Edge Distr.** in **View** menu to see distribution of distances of detected objects to the nearest point in the outline. After that you can save TXT file where pairs "coefficient of lateral diffusion" – "distance to nearest edge" values for all objects detected in this record will be saved for future analysis.

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Settings

Max Radius

Max Radius (in pixels) – Is the maximum search radius used to link objects between frames. The tracking algorithm needs to link objects between adjacent frames so that they are made into contiguous "tracks" in space and time. If when searching a subsequent frame no object is found within the Max radius (using the current object centroid as the centre value) then the object track is terminated. This parameter limits the maximum "jump" in spatial coordinates that an object makes between adjacent frames and depends upon frame rate and object speed. Max Radius should be larger than the expected object "jump" distance between adjacent frames. Otherwise, tracks will be erroneously terminated. Furthermore, Max Radius should be significantlysmaller than the average distance between neighbouring objects on a given frame otherwise unrelated objects will be erroneous linked on subsequent frames. The experimentalist might need to juggle imaging conditions to satisfy these criteria.

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Track Length

Track Length (in frames) – If a tracked object is terminated before a minimal number of data points (Track Len) then the track is discarded. It is often helpful to keep this value above a certain limit (perhaps > 10) because short tracks can be generated spuriously by noise. Whereas longer tracks are more reliable for future statistical analysis (e.g. for constructing MSD versus dT plot) – You may instead prefer to keep all track lengths (>2) and then filter the data at a later point in the analysis.

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Q-Threshold

Q-Threshold – This parameter determines the minimal value of *Q* which is used to detect single particles on the images (see Find Single Particles for further information). Note: The Q-Threshold value displayed is 10 times bigger than the *Qmin* used for calculations. The multiplied *Qmin* value will also be appended to the GMI filename as a tracking parameter. This is done to avoid using floating point numbers. Note: If Q-Threshold is low (e.g. < 10 *Qmin*<1.0)), Track Length is short, and Max Radius is big then you will find that in noisy records some erroneous, noise-generated, tracks will appear and the tracking algorithm will run slowly.

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Cluster Threshold

Cluster Threshold (counts/pixel)– In some cases small bright objects might occur due to clusters of fluorophores. To exclude these from analysis the Cluster threshold should be set above average single-object intensity but below average cluster-object intensity. Objects with intensity higher then Cluster Threshold will be discarded during**Tracking**.

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Link Tracks in Batch Mode

Link Tracks in Batch Mode – Leave this line checked if you want to link tracks which had one data point missing during Batch Analysis. It is important to use Add Blank Frames option if you use Fuse Records for analysis and want to link tracks in them. You also can link tracks at any time after you done the Batch Analysis when you load GMI file with the results of tracking (**Data → Link Tracks**).

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Show

Kdiff Tracks

Click **Static Tracks [S]** after you complete Detect and Tracking procedures to show trajectories

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Static Tracks

Click **Static Tracks** after you complete Detect and Tracking procedures to show trajectories of detected objects on image. The tracks are static and will be drawn on every frame in the record even if the object itself is not present on this particular frame. Click**Static Tracks [S]** again to remove the trajectories from the image. You can change Track/Cross Color inSettings Menu of the main window.

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Tracks in time

Click **Tracks in Time [T]** after you complete Detect and Tracking procedures to show trajectories of detected objects on image. The track for the particular object will appear on the frame where the object was first detected and will be drawn to the position of the object on current frame. The track disappears when the object is not detected anymore on the current image. Click **Tracks in Time [T]** again to remove the trajectories from the image. You can change Track/Cross Color in Settings Menu of the main window.

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Prev. Spot

Click **Prev. Spot [PgUp]** after you complete Detect and Tracking procedures to show one of the processed spots properties in **Measurement and Statistics (M&S)** window. It can be MSD versus dT plot of given object or its intensity or X and Y coordinates in time according to the chosen property in **Show** menu of M&S window. You can see other detected spots data pressing [PgUp] and [PgDn] keys on the keyboard. The blinking trajectory on the image will correspond to the spot shown in M&S window. You can save the data for any particular spot using options in **File** menu of M&S window.

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Next Spot

Click **Next Spot [PgDn]** after you complete Detect and Tracking procedures to show one of the processed spots properties in **Measurement and Statistics (M&S)** window. It can be MSD versus dT plot of given object or its intensity or X and Y coordinates in time according to the chosen property in **Show** menu of M&S window. You can see other detected spots data pressing [PgUp] and [PgDn] keys on the keyboard. The blinking trajectory on the image will correspond to the spot shown in M&S window. You can save the data for any particular spot using options in **File** menu of M&S window.

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MSD versus dT

Click **MSD versus dT** after you complete Detect and Tracking procedures to show averaged MSD-dT plot in **Measurement and Statistics (M&S)** window for all the objects tracked in this record. In fact this window will appear automatically when the software finishes Tracking procedure. You can exclude individual objects from the data set by browsing through spots using [PgUp] and [PgDn] keys and mark any spot as “Bad” pressing [Enter] key. You also can unmark it pressing [Enter] again. The “Bad” spots will not be used for averaged MSD versus dT plot and will not be saved as data in GMI or TXT files. You can save the data using **File** menu of M&S window. You are advised to use satellite software “Motility” if you want to sum data from few similar records or do future filtering/other analysis on the data. Please contact G Mashanov if you need some advise how to use Motility.

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Data

Save X-Y-Int Data

You can use **Save X-Y-Int Data (GMI)** option to save the results of ASPT in a special file format (GMI) which keeps all the data (trajectories) in the numerical form. This data can be loaded again (Read X-Y-Int Data (GMI) eliminating the need to process the same record again. You are advised to use satellite software "Motility" if you want to sum data from few similar records or do future filtering/other analysis on the data. Please contact G Mashanov if you need some advise how to use Motility. Please note that tracks marked as "Bad" wil not be saved in GMI file. Use [PgUp] and [PgDn] keys to navigate though individual tracks and mark it as "Bad" by pressing [Enter] key on keyboard.

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Read X-Y-Int Data (GMI)

Read X-Y-Int Data (GMI) - The results of tracking can be loaded again into ASPT module eliminating the need to process the same record again. Please note, that the corresponding GMV file must be open in GMimPro, otherwise the error messages will appear and ASPT data will not correspond to the GMV record currently open in GMimPro.

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Save Distributions as TXT

Save Distributions as TXT - You can save distributions on left and right panels of ASPT window in TXT file to import it into statistical package like Excel for future processing.

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Save Tracked Data as TXT

Save Tracked Data as TXT - You can save all tracks X, Y, and intensity data in TXT file to import it into statistical package like Excel for future processing. Please note that tracks marked as "Bad" will not be saved in GMI file. Use [PgUp] and [PgDn] keys to navigate though individual tracks and mark it as "Bad" by pressing [Enter] key on keyboard. Please check **Include Time in TXT File** line if you want to include time stamps in TXT file. By default the tracks saved in rows, but the size of the row is limited to 252 values, which means that the data will be truncated if tracks are longer 252 points. Please check **Data in Columns** line in **Save Tracked Data as TXT** submenu if you wish to save tracks in columns which are not limited by track length. However in this case the number of tracks is limited. This number will depend on if you are saving X and Y coordinates OR X and Y and Intensity data OR Intensity data only. If you save TXT data in columns the beginning of the track will correspond to the frame (row) where the object was first detected, unlike saving data in rows where all the tracks starts in the first column to spare the size in the row. By default X and Y coordinates are saved in micrometer units (relative to the pixel where the trajectory begins (0.0, 0.0). If you need to know the position of the object in pixel coordinates (relative top-left corner of the image) check **Use Pixel Coordinates** line in **Save Tracked Data as TXT** submenu before you save the data. The data will be saved with 0.001 pixel precision but the actual precision will depend on tracking parameters.

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Manual Tracking

Overview

Manual Tracking – This is an additional module which can be used to track limited number of objects by hand. First - you have to create a new object (click **New Object**). This object will be active and ready for tracking. Second - you move to frame where you want to start tracking and place your cursor (cross) at the position where tracked object is located. Click left mouse button. The position will be marked and software will move to next frame where you move cursor over the object and click again. Repeat this operation to the frame where you want to stop tracking. Coloured track will show location of this object at different time points. You can move forward and back along the record and correct the track by clicking on the new "correct" position. You can delete data points by clicking right mouse button – it will delete object's mark on previous frame and rewind record one frame back. You can create next object (**New Object**) and do tracking for next object. Current object which you can track at the moment is marked by blinking square on its button. Click on the object's button to select it (avoiding square). If you click on the square in the button you will make this object active or inactive. Diagonal cross is indicating the state of an object. Active objects are used to calculate average velocity and velocity distribution. Only active objects will be saved (**Save Data**) for future analysis. Active objects are represented by bold lines on the image and velocity graph, inactive ones by thin lines. Velocity graph shows up to ten "time" – "velocity" graphs closest to currently selected object (blinking cross). Select (click) another current object to see other graphs. Coloured dots on right side of the buttons will show which velocity graphs are displayed at the moment. You can change a graph's properties or print it by putting the cursor over it and pressing right mouse button. Average object's velocity is shown on the object's button. Average velocity/ standard deviation and (number of data points) will be displayed in the hint label when you place mouse cursor over particular button. If number of objects is bigger the panel size then the Scroll Bar will appear on the window to navigate on the panel. Note: You can not select ROI or plot Profile when Manual Tracking window is open because both mouse buttons assigned to tracking functions.

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Save Data

Save Data - You can save the results of tracking as GMI file **Save GMI file** for future analysis or you can read the GMI file back to continue tracking. You can load it into ASPT module to analyse the data there. You can also save data in TXT file: velocities measured at every step for all active objects (**Velocity Data)**, or save average velocity, SD, and number of points for every active object (**Object's vel/SD/N)**, or distribution of velocities (**Distribution of Vel.)**, or each XY point marked during record (**Each XY point)**. Note: all data in TXT will be in micrometer/s and micrometer units.

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Index

- -

- 3 -

- A -

- B -

- C -

- D -

- E -

- F -

- G -

- I -

- K -

- L -

- M -

- N -

- O -

- P -

- Q -

- R -

- S -

- T -

- V -

- Z -