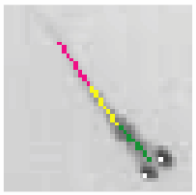
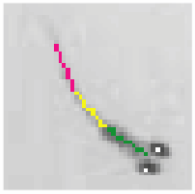
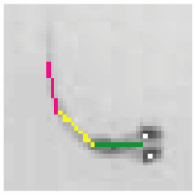
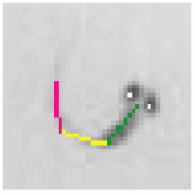
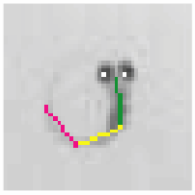
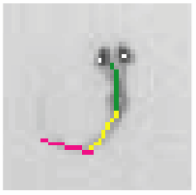












Flote v2.0

Biological Tracking Software

by Harold Burgess and Michael Granato

Index

Introduction

Flote is a software application for the high-throughput behavioral analysis. The main purpose of this utility is to take stacks of images of groups 5-8 day old zebrafish larvae recorded with high speed cameras and detect the initiation of discrete elements of the larval motor repertoire.

In the process, Flote extracts a large amount of information about the kinematics of movement, which can also yield useful insights into behavioral modulation. Flote can also be used for the much simpler application of tracking larvae and measuring non-kinematic features, like position, speed and orientation.

The algorhythms underlying Flote are described in these two articles:

Burgess HA and Granato M. (2007) Sensorimotor gating in larval zebrafish. J Neurosci. 27:4984-94.

Burgess HA and Granato M. (2007) Modulation of locomotor activity in larval zebrafish during light adaptation. J Exp Biol. 210:2526-39.

Flote was written by Harold Burgess in the laboratory of Michael Granato at the University of Pennsylvania. Much thanks to John Crocker from the Department of Bioengineering for the particle identifying algorithm, Jeremy Magland for mathematical assistance and Hannah Shoch for extensive road-testing.

Bugs, both known and obscure remain in the code. Please let us know if you find anything egregious. In addition these instructions are a work in progress, incomplete in some areas.

1. Installing the software

1. 1. Installation

Flote is written in the IDL programming language. To run Flote, you must have the IDL Virtual Machine installed on your computer. This is a free download, which (currently) may be obtained from:

http://www.ittvis.com/download/download.asp?searchstring=&radDownloadType=Download&ProductVersion=279&Platform=All+Platforms&DownloadType=All+Types&submit=Submit

In case the address of the download site changes, search for 'ITT Visual Information Solutions' and find the link on their website for 'IDL VM'. Note that the IDL download is going to install two pieces of software on your computer (1) IDL itself which will operate in 'trial mode' for 7 minutes and (2) the free IDL runtime platform which will execute pre-compiled code. To use Flote you only need the runtime platform and therefore do not need to purchase IDL.

In principle, the code should run under any platform for which an IDL Virtual Machine is available, however we have not attempted to use anything other than Windows 2000 and Windows XP. The minimum hardware we have used is 512MB RAM with a 2.8 GHz processor.

Once you have installed the IDL Virtual Machine, make a directory for the three files, 'flote.sav', 'flote_setup.sav' and 'batchan.sav'. Run the 'flote_setup.sav' file. Note if you have software like SPSS installed on your computer, you will need to right click on the flote_setup.sav file and open it with ' IDLRT'. This will create a folder: 'C:\ftrack\' with various subfolders, used for storing your settings and in some cases your output files.

In addition, you need to specify three settings:

1. The default video directory. This is where Flote will by default look for video files.
2. The save tracks directory. This is where Flote will by default save tracking files. It is often advantageous to run Flote on multiple computers to speed up the tracking. If you intend to do this, make sure that the 'Save tracks' directory is accessible from all computers. The exact path to the directory should be specified. For example for a local directory it might be 'C:\Share\tracks' and while the path to the same directory from another computer might be 'S:\tracks'
3. A computer identifier. This is only really important if you will use more than one computer for analyzing data. Each computer should have an name.

When you have entered your settings, press the **Save** button then **Quit**. Alternatively, press Quit to leave the setup program without modifying the settings.

By default, when you open Flote, it displays 4 larvae in the middle of the screen. To change this, place a jpg image in the 'C:\ftrack\startim' directory.

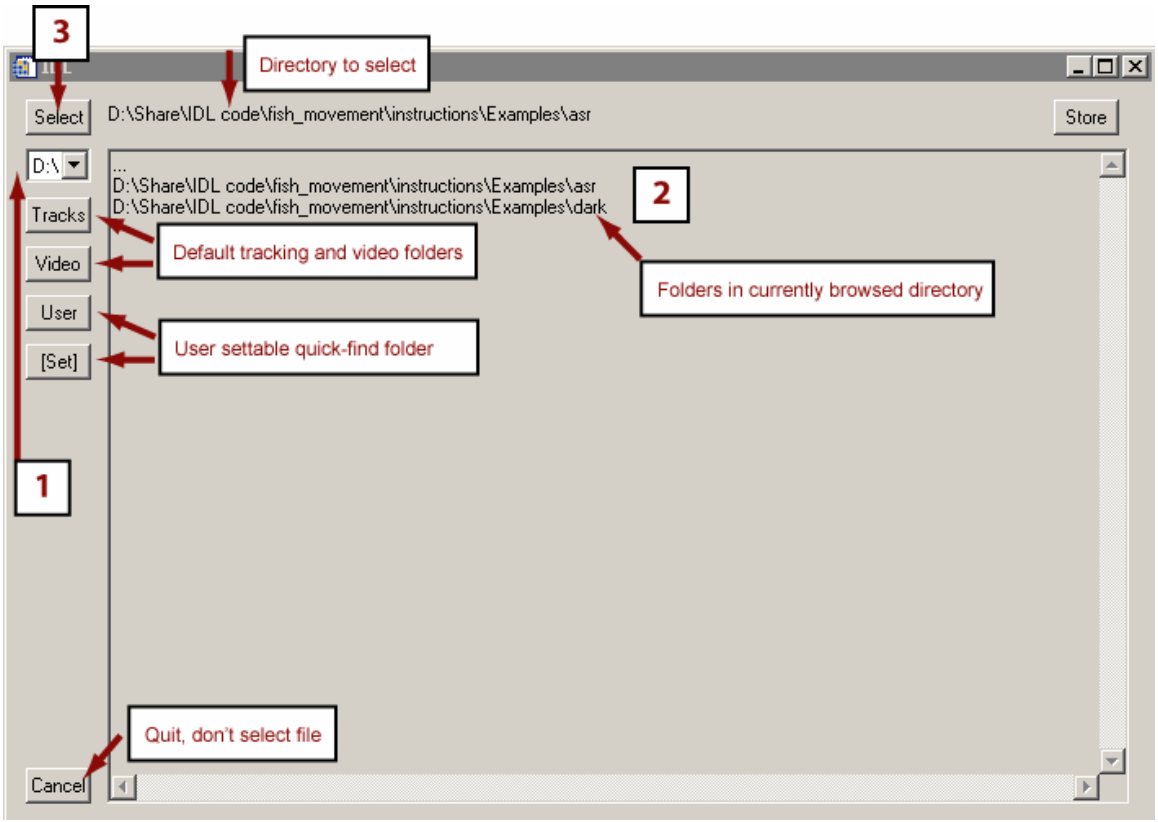
1. 2. Un/Re-installing

To uninstall, just delete the directory 'C:\ftrack' and the Flote.sav files.

For a completely fresh install, delete the directory 'C:\ftrack' and the old Flote.sav files, then run the flote_setup.sav again.

To upgrade to a newer release of Flote (and keep most of your saved configurations), don't delete the C:\ftrack directory. The flote_setup.sav program will overwrite only your Default configuration and default paths. This procedure is also useful for giving your computer a new name, or changing the default video and tracks directories. Occasionally, depending on the version you are upgrading from, this may not be sufficient and you should then perform a fresh installation.

1. 3. Using the Folder Browser



1. Select the drive you want (or use the 'Tracks', 'Video' or 'User' presets to quickly navigate to the target folder)

2. Navigate to the desired folder. Double click on a folder to enter it. If you enter a directory with a huge number of files, it can take a long time to enter the folder (especially if they are on a network drive) - avoid this! Double click on the '...' to go back up a directory. Single click on a folder to set it as the folder which will be selected. You can always see which folder is the one which will be selected, by the text beside the 'Select' button.

3. Press 'Select' to choose the folder beside the button.

To cancel the operation, hit 'Cancel'.

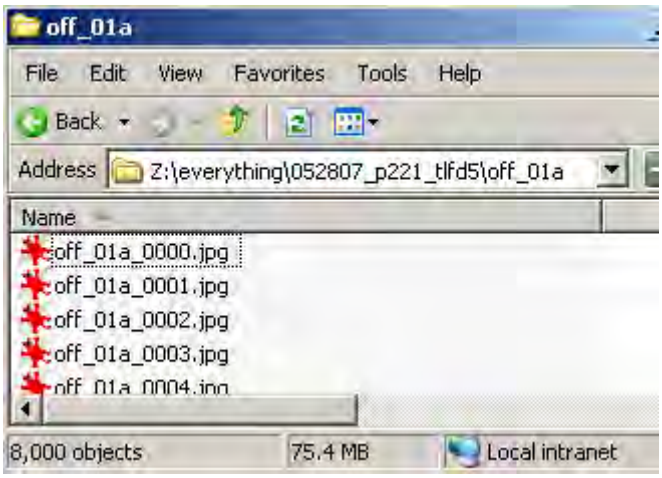
The 'Tracks' and 'Video' folders are the default folders for storing video and tracks specified during installation. A third preset 'User' is available. You can set this by pressing the '[Set]' button when you have choosen a desired folder. Occasionally you will have another option on the top right 'Store' - this sets the folder as the default for the current type of folder selection operation.

2. Recording Videos

2. 1. Kinematic Analysis of Zebrafish Larvae

Larvae should be recorded using a suitable lens so that their length is roughly 50 pixels. Flote will not work well unless the larvae are of about the same length. Older larvae (after about 10 dpf) are often variable in length, so either presort ones of about the same length or use younger larvae.

The recording speed will depend on the application. However, for automatically identifying motor patterns, the software requires that the recording is made at exactly **1000 frames per second (fps)**. We generally a resolution of 512x512. Videos should be saved as a stack of **8-bit** (i.e. grayscale) JPEGs with **75% 'quality' compression**. Lower quality compression (< 50%) leads to significant degradation in tracking. Higher quality compression (>90%) yields much larger file sizes resulting in reduced tracking speed and produces only marginal improvements in accuracy (less than 1% more 'trackable' objects).



Each video should be saved in its own folder and the names of the file stack should be the same as the **folder name PLUS an underscore PLUS the frame number**. The first frame should be numbered zero.

Example: You recorded a 8000 frame video, which contains 40 events each of 200 frames. Make a folder called 'off_01a' and inside the folder, save the video as a stack of JPEGS where the first frame is called 'off_01a_0000.jpg' and the last frame is called 'off_01a_7999.jpg'

If your recording software doesn't permit this naming convention, videos will still load, so long as the stack of frames are still in their own folder and in alphanumeric order. However image stacks will take longer to load.

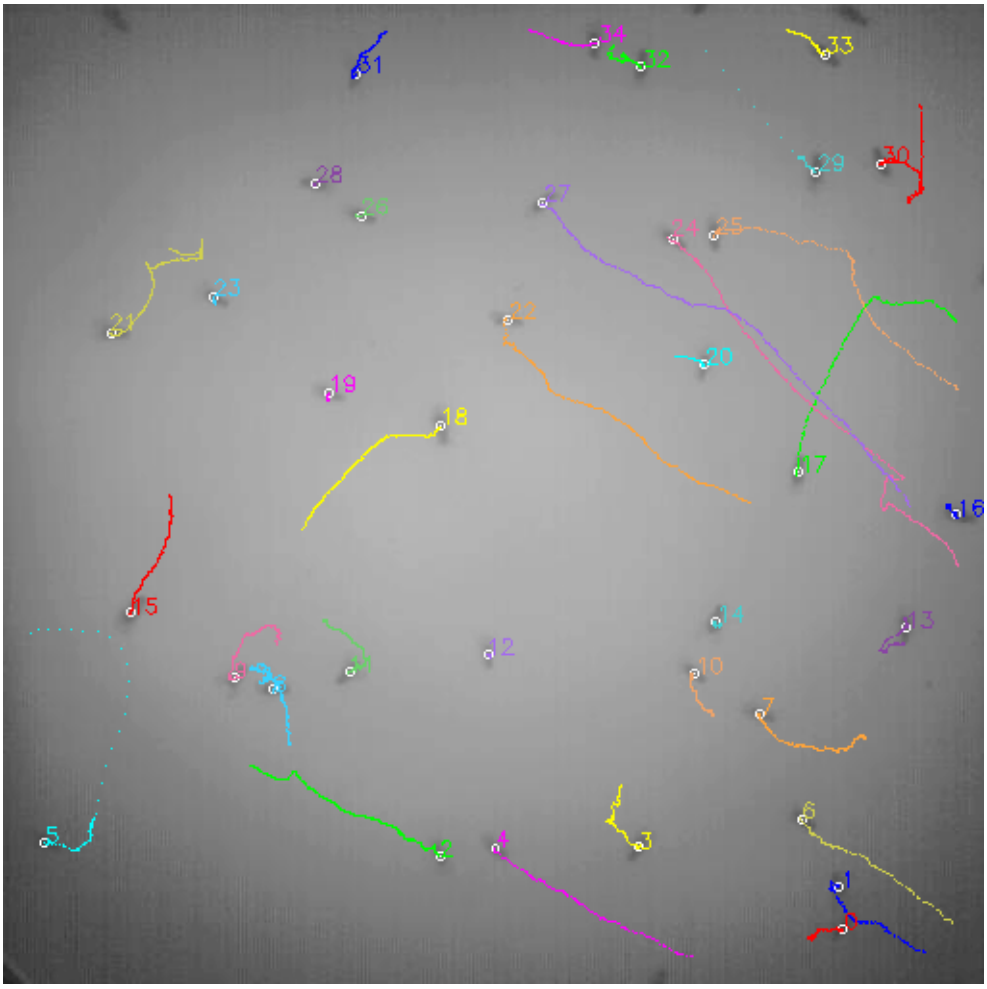
2. 2. XYA Analysis of Zebrafish Larvae

Flote can operate in a simpler mode, in which only position and orientation information is analyzed. For this type of recording, it is still necessary to optimize centroid finding (see below), but not essential to optimize eye-finding or curvature. Resolution is not

important, so long as the position (and usually orientation) can be succesfully determined by Flote.

2. 3. Other animals

Flote can also perform simple tracking of other groups of animals, yielding position, speed and orientation information. Where displacement is a reliable measure of movement, it can also classify individuals as moving or stationary. For robust tracking, the recording frame-rate should be sufficient that individuals move less than half a body length between frames. For Drosophila, we find that 100 frames per second works well.



3. Setting object detection parameters

3. 0. Loading Videos

Choose: File→Select video directory then single click on a folder containing a stack of JPEGS. Click 'Select'. The first image in the stack should now be displayed and the message window will display 'First image in folder' and the dimensions.

If there is an error, the message window may read 'No suitable jpegs in folder'. This usually reflects non-conformity of the filenames to the convention described in section 2.1, but could also reflect an unusual mode of saving jpegs - remember they should be grayscale. There is a known bug in Flote - if you try selecting a new working directory, while the graphing window is open it will also generate an error. Just close the graph window and try again.

For optimal loading, the first file should be numbered zero. However if the video directory contains a '.cih' file, Flote recognizes the directory as originating from a Photron camera. These video files are saved with the first file as _000001.jpg instead of number zero. For such directories, Flote will automatically add +1 to the file number. However in telling Flote which files to load, you should still regard the first frame as being zero numbered.

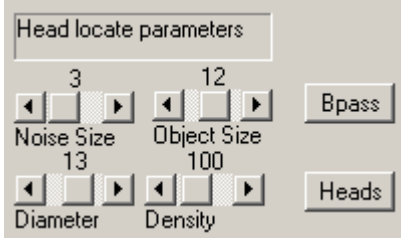
If you were unable to save using the convention 'filename'='folder_XXXX.jpg' then toggle the 'File→Alphanumeric file order' to be checked. Flote will assume that the file stack is simply saved in alphanumeric order. When it loads a video, it will each time make a list of all files in the directory and open the appropriate subset. This is slower than when it can assume that frame numbers are appended to file names, with the first frame being zero. **If you have any trouble loading videos, the first thing to try is applying the "Alphanumeric file order" setting**.

If there are a large number of frames in the directory and/or the directory is hosted on a remote server, it could take around 30 seconds for the 'Select directory' window to close and the first file to display.

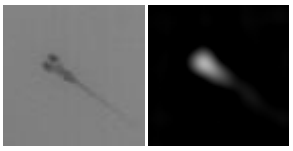
Note that images that are not 512x512 are stretched to fit the display. This may affect the display of tracks and other visual annotations, but does not affect the actual analysis.

3. 1. Finding the head centroids.

The 'Head Locate' part of the main window allows you to adjust parameters for finding the head centroids.



Click the **Bpass** button. This shows the bandpass filtered image that will be used to find head centroids. The bandpass controls are the 'Noise size' and the 'Object size'. Objects smaller than the 'noise' size (in pixels) or larger than the 'object size' will be smoothed out. For larval fish whose length on the image is about 50 pixels, using a noise=3 and object=12 sufficiently blurs the eyes and head into a single object.

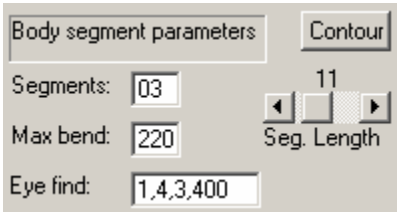


Now play with the 'diameter' and 'density' functions. These are thresholds. Only objects whose diameter and intensity respectively are larger than these numbers will be recognized as head centroid positions. For larval fish recorded at the recommended image size, diameter=13 usually suffices. Density will depend on your illumination. We generally use density=100.

To check your settings, make sure that the parameter Display→Head Positions is on, then click 'Heads'. All the fish should be recognized, with a crosshair showing the position of the head centroid for each fish.

For more information on the particle identifying algorithm implemented see: Crocker, J. and Grier, D. (1996). Methods of Digital Video Microscopy for Colloidal Studies. Journal of Colloid and Interface Science 179, 298-310.

3. 2. Finding the body curvature

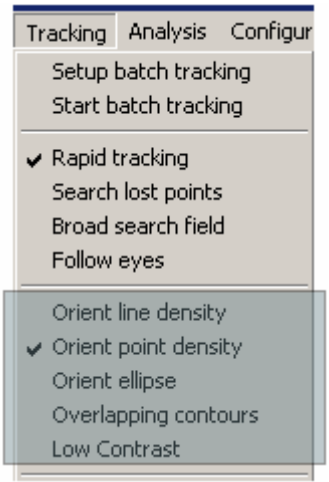


The body segment part of the main window contains controls for changing how the larval curvature is modeled.

The body is modeled as a series of (at least) three segments. The number of segments can be set in the 'Segments' field. For tracking larval fish, we use 3 segments. The length of the segments is set using the 'Seg. length' slider bar. Two factors are important - the first segment should end just before the caudal extremity of the swim bladder. The head begins to bend at around this point, so the segment length needs to end here. The second factor is that depending on your contrast, the tail will begin to thin out and be hard to recognize. If the segments are too long, the third segment will be 'jumpy'. Generally a segment length of 11 or 12 is suitable.

The 'Max bend' parameter is not generally changed - its the segment of the circle searched for the 'next segment'. This is set at 220, giving a maximum segment to segment angle of 110 degrees.

There are four methods for finding the segments, which can be selected under the Tracking menu.



Orient line density - the default and usually most reliable. This method measures the density of bars radiating from successive initiation points and identifies the most intense bar as the continuation of the body axis.

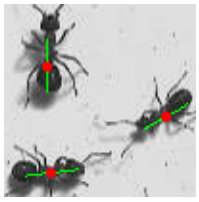
Orient point density - Searches only for the most intense point at the end of bars radiating from successive points on the body. This is sometimes more accurate in images with noise, for example when larvae are gridded in a high contrast array.

Orient ellipse - Fits an ellipse to the centroid using David Fanning's fit_ellipse routine (www.dfanning.com) and extracts the orientation of the major axis of the fitted ellipse. The ellipse is fit using a box around the centroid whose width is specified by the 'radius' slider. This is not intended for larvae fish, but is quite good for measuring eye movements during optokinetic assay.

Overlapping contours - Uses overlapping bars to measure body curvature. This is not useful for larval fish and is intended to allow measurement of the orientation of other organisms. The differences are that the first orientation bar is centered on (not emanating from) the centroid. Consequently the orientation range is from 0 to 180 degrees not a full 360 degrees.

Other examples:





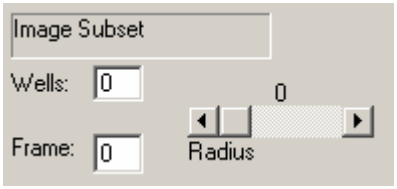
In addition there is a Low Contrast option in the 'Tracking' menu. If your light source is weak and contrast is poor, low contrast tweaks the line density algorithm to improve curvature analysis.

3. 3. Finding the eyes

The 'Eye Find' parameter is by default '1,3,3,400,12'. These numbers refer to the parameters passed to the bandpass function for finding eyes: noise size=1, object size=3, diameter=3, density=400, search radius=12. To check that these parameters work for your video, set the 'head locate' parameters with these settings. While the whole video field will probably become covered in 'head' check marks, there should be marks clearly identifying every eye of every fish. In poor illumination you might need to reduce the density to 100 for the eyes to be correctly found. If the image of the larvae is small, you might need to reduce the search radius. The search radius needs to be larger than the object size.

By default, Flote only finds eyes in the first frame - this is useful to exclude larvae which are lying on their side or contrast elements which are not larvae. However, by finding the eyes in every frame, Flote can also determine whether the larvae maintain an upright posture throughout their movement. This slows down tracking, but can be enabled by selecting Tracking→Follow eyes.

3. 4. Setting image regions



Often you will want to exclude contrast elements at the edge of the image, for example the edge of the recording arena. Two options in the 'Image subset' area allow you to do this. The 'Frame' field lets you specify a number of pixels from the edge to exclude - by default its set at 0. The 'Radius' slider excludes points outside a circle, centered in the middle of the image, whose radius is half the image width less the value of the radius slider. This is useful if you are recording inside the whole area of a Petri dish.

The 'Wells' field lets you divide the field into an X by X series of sub-fields, where each sub-field is expected to contain just one fish. For example, set 'Wells' to 2 and press the **Heads** button. You'll see a 2x2 grid on the field. During tracking, each of the four squares is assumed to contain a single fish. If multiple elements are identified, only the darkest object will be analyzed. This is useful if the subdivisions correspond to individual wells in which fish have been placed. It is often difficult to prevent the 'Heads' algorithm from identifying parts of the well as larvae. However, so long as the larvae is the darkest object in the sub-field, it doesn't matter, only that larva will be analyzed. By default Wells should be set to '0', indicating that there is no division of the field and that every fish identified should be analyzed.

If you set 'Wells' to 1, only one fish in the whole field will be analyzed. This can be very useful if you are tracking adult fish where both the head and the swim bladder are typically found as 'heads', but the actual head is darker.

3. 5. Excluding false contrast elements

Contrast elements are often misidentified as larvae - excluding these is critical to getting good tracking analysis. Ensure larvae are in very clean water with no dirt floating around. Avoid imaging the edge of the testing arena. Once this is optimized, the main three tools you have against this problem are:

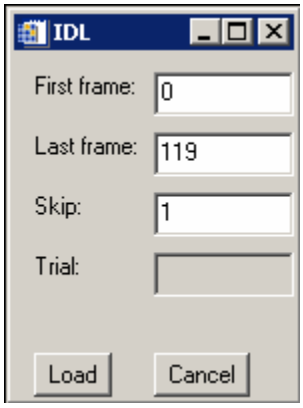
1. Setting the image regions to exlude regions with false contrast elements (see 3.4)

2. Adjusting the head centroid parameters so that false contrast elements are not identified as fish

3. Adjusting the eye-find parameters so that false contrast elements which are identified as fish, do not have 2 eye spots identified. These elements are then later able to be excluded from analysis.

4. Tracking a single video

4. 1. Loading the Video



Load in the frames you want to track. Select File→Load subset and in the box that appears enter the number of the first frame in the trial, and the number of the last frame in the trial. The option 'skip' allows you to only load every Xth frame. In general leave this at 1 - all subsequent kinematic analysis for larval zebrafish requires videos to be performed at 1000 frames per second.

For example, if each trial was 400 frames, and the folder contains 8000 frames (20 trials of 400 frames) and you want to look at the third trial, enter 800 beside 'first frame', 1199 beside 'last frame'. Then click **Load**.

Alternatively, if the each folder contains only a single trial (for example a 1 second long recording of 1000 frames), after selecting the working directory you can choose File→Load whole directory and the full 1000 frames will be loaded. Memory is limiting but a computer with 2 Gb memory should be able to load and track at most around 2000 frames. Typically we analyze 100-400 frames (i.e. 0.1 to 0.4 sec).

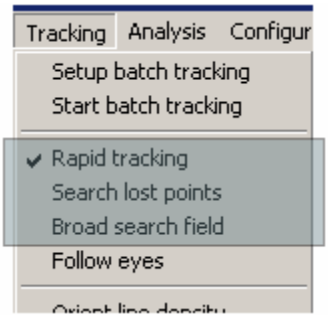
Large videos, especially when transferred from a remote computer can take several seconds to load. You can see the progress of loading a video in the dialog box above the image.

Check that the video has been loaded correctly. The slider bar above the image shows the frame number being displayed. If you move the slider bar, the frame number should change and the appropriate image displayed. To play the whole video, press the **Fwd** button to the right of the slider bar (and to play it backwards, press the **Back** button to the left of the slide bar). The **frame-rate** slider sets the 'skip' - by default its set at 5, meaning that playing the video will display frame 0,5,10,15 etc. For very long videos, you will likely want to adjust this to 10 or 20. For saving videos (see section,) you will want to set this number to 1 so as to save every frame in the video.

When you are finished analyzing a given trial, you can select File→Next subset or click the **Next** button to retrieve the next trial. Conversely the **Prev** retrieves the earlier set of frames. Example, if you loaded the 400 frames from frame 800 to frame 1199, selecting the next trial would load frames 1200-1599, while selecting the previous trial would load frames 400-799.

Once you have loaded the first video, the File→Load subset menu allows you to enter a trial number. If you specify a trial it over-rides any text in the 'first frame/last frame' box. For example, if each trial is 120 frames, and you select trial 12, then frames 1440 to1559 will be loaded.

4. 2. Setting Tracking Options



There are two methods of tracking, selected by toggling the Tracking→Rapid tracking setting. The 'slow' method (option unchecked) is a simple implementation of the tracking algorithm of Crocker and Grier. The 'rapid' method (option checked) uses a similar approach (connecting nearest-neighbors across frames), but simply institutes a local search across frames, after identifying initial object positions. For all applications we have tried, the 'rapid' method is as accurate and much faster. It is possible that in a very crowded field, the 'slow' method would work better, but in general keep Rapid tracking on. Rapid tracking has a maximum allowed object size of 25, or 50 if the 'Broad Field Search' is checked - for larger objects the slow method is necessary.

There are two other options under the Tracking menu, both of which are left unchecked by default. They only work under Rapid tracking mode. These options are not useful for most applications and should usually be left unchecked.

Search lost points - if the larvae becomes fainter during the trial (for example, due to waves of water passing across it or fast movement introducing a slight blur) the density threshold will be automatically reduced until the particle is located again. Having this option on can greatly slow down tracking - however the fact that it is doing so means that more larvae are being successfully tracked. You can try reducing the initial 'Density' threshold to prevent larvae being lost.

Broad search field - by default, during rapid tracking, a local search for the nearest object in a 50 pixel box around the position of the object in the last frame. If this box is checked, the search is expanded to 100 pixels. This is useful when:

1. Tracking is being performed for reasons other than kinematic analysis, where the recording speed might be less than 1000 frames/second, and the larvae move further between frames.

2. You need to set an object size greater than 25. With the 'Broad Field Search' checked, you can have an object size of up to 50.

Rather than manually set the object detection and tracking parameters every time, once you are happy with the parameters, you can save the configuration by selecting Configuration→Save this config. Give the configuration a name and press 'Save'. To

reload the configuration, either select Configuration→Load Tracking Config or Configuration→<*Saved Configuration Name*>.

4. 3. Tracking a video

With a video loaded and all options set, click the **Track** button. In the dialog box above the video, you should see the progress of the tracking. First the object positions are found and linked into tracks. Second the orientation of each object is determined across frames. Tracking is finished once all objects have been oriented - for example the dialog will read 'Orienting item 3421/3421'.

If you are going to load and track many trials, it is convenient to set the File→Track on Load option. When set, after pressing the **Next** or **Prev** buttons, Flote will automatically track frames after they have been loaded.

4. 4. Displaying tracked data

The Display menu controls how the video is displayed on the screen, annotating it selectable options.

The following options are toggle switches, turned on and off by selecting the option:
Tracks - displays the path followed by each object.
Head positions - displays the head locations.
Eye Positions - displays the eye locations.
Head Orientation - displays the first (i.e. head) segments.
Body Contours - displays all the segments.
Background - displays the video (leaving the annotations intact).
Number larvae - displays the number assigned to each object.
Neighbours - displays the nearest neighbour to each object.
Orientation trail - shows a 'trail' of the orientation in the previous 10 frames.

The Head Shape submenu contains various options for designating the head centroid.
The Eye Shape submenu contains various options for displaying the eye positions

Selecting Setup Colors opens a window in which the color assigned to the head, eyes, tracks, numbers and three body segments can be controlled. The Multicolor tracks toggle over-rides the color assigned to the larval number and track to give each larva a distinctive color. The Grayscale toggle over-rides any color selections with a default black and white palette.

The Selected Object submenu controls how the selected object is displayed:

Zoom and Center - Toggles whether the zoom window keeps the head centroid of the selected object in the middle of the window.
Highlight - Toggles the display of a circle around the head of the currently selected object.
Mask others - Toggles the display of the head and body segments of all objects, or just the selected objects.
Fix Rotation - Rotates the zoom window so that the head of the selected larva is always facing to the left. This only operates if the 'Zoom and Center' has been selected.

Copy to file - Toggles saving of the image to an output file. Every time the image is changed, the current video display is saved to a '.tif' file in the 'C:\ftrack\images\' folder. This is very useful for making annotated videos. Simply toggle this on, then press the **Fwd** button beside the slider at the top of the image. Every frame will be annotated and saved in order. Remember to turn this option off! If there are files already in the 'C:\ftrack\images\' folder, new files will be numbered after the existing files.

Annotate Time Series - Flote can annotate the time series data onto the main window and/or the zoom window. First toggle on Display→Annotate→Time series then select the main or the zoom window to be annotated with the time series data this is currently being plotted (see section 6.) It is not possible to select a subset of the time series - every frame is included in the annotation.

Annotate Stimulus - It is sometimes useful to represent the frames in which stimulus conditions changed. To do so, toggle the Display→Annotate→Stimulus, then select the main or the zoom window to be annotated. Next choose Display→Annotate→Setup stimulus and enter the first frame and the last frame to mark the stimulus.

To make the annotation larger, for example if you are planning to save frames and compress them subsequently to make a movie, toggle on the Display→Annotate→Double Thickness option.

If 'Use External' is selected then external IDL code is called to annotate the image. This is for future use only.

To zoom on part of the window, hold down the right mouse button and drag the mouse across the area you want to highlight. A zoom window will appear, with an expanded view of the region. The physical size of the zoom window is fixed on the x-dimension (i.e. the zoom window is 500 pixels wide). So if you select a small horizontal region for zoom, the magnification will be large. The y-dimension of the zoom window is simply scaled proportional to the scale factor for the x-dimension.

You can select a 'standard' zoom window dimension by checking one of the options in DisplayDisplay→Zoom Size. This will magnify a window of 100x100 or 200x200 pixels into the 500x500 pixel zoom window. The top left corner of the zoom box will be where you right-clicked.

After tracking, you can right click on a larva to highlight it and a zoom window will appear with the selected larva in the center. Doing this automatically sets the Zoom and Center option, so that the larva will remain in the center of the zoom window while it moves.

Left clicking inside the zoom window will give you the pixel location in the message window. Dragging with the left mouse button depressed gives the dx, dy values and distance in the message window. This can be useful for making length measurements. Right clicking inside the zoom window puts the selected point in the center.

Note that when Copy to file is toggled on, the zoom window will also be saved. This is useful for making enlarged videos of a part of the field.

4. 5. Saving tracking information

You can save the tracked information for a video by selecting Tracking→Save Tracking File. A file containing the position and orientation information will be saved in your default 'save tracks' directory (see section 1), with the file name 'track_eXXX.sav' where the XXX corresponds to the number of the video in the directory.

Tracking files are proprietary IDL data files that can't easily be opened or viewed. To convert these files to tab-separated text files (easily opened in Excel) select Tracking→Convert tracks to text. Choose the directory containing the track files and press OK. Files in the selected folder will be copied to the 'C:\ftrack\' folder, with a '.txt' suffix. These files contain 10 columns. Each row specifies the position and orientation of a single object in a single frame of the video.

Fish - the number assigned to each object in the video
Frame - frame number for that object
x - x position of the object in that frame
y - y position of the object in that frame
mass - density of the object
radius - a measure of the object size
eccent - a measure of object symmetry
orient1 - the orientation of the first (i.e. head) segment of the object
orient2 - the orientation of the second (i.e. midbody) segment of the object
orient3 - the orientation of the third (i.e. tail) segment of the object

5. Batch tracking multiple trials

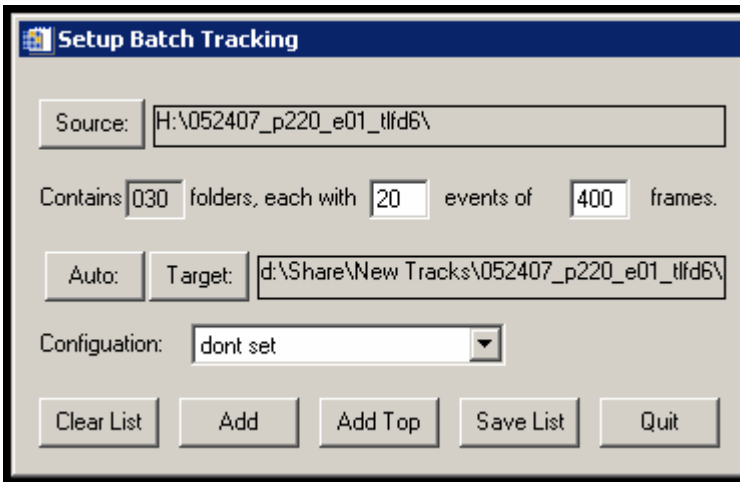
Flote is designed to automatically track multiple trials. First, to be clear on terminology. By 'trial' or 'event' we mean a single short video (typically 100-400 frames) which records the response of the fish to one stimulus. Generally, a given group of fish are exposed to multiple stimuli. Flote expects that a folder containing a stack of JPEG files with continuous numbering, in fact represents the multiple trials.

For example, folder 'DRUGA_P01' contains files 'DRUGA_P01_0000.jpg' to 'DRUGA_P01_7999.jpg'. In this case, we have tested drug A, with plate #01 of fish. The 8000 files might be 80 trials of 100 frames per trial, or 20 trials of 400 frames per trial. All these need to be individually tracked, resulting in 80 and 20 tracking files respectively.

'DRUGA_P01' was likely only a single data point of the whole experiment. Thus, the folder 'DRUGA_P01' itself is inside a larger folder containing all the data for a given experiment. For example, you have a larger folder 'Startle_05212006' containing six folders 'DRUGA_P01', 'DRUGA_P02', 'DRUGA_P03', 'CONTROL_P01', 'CONTROL_P02' and 'CONTROL_P02'. Each of the six folders contains 8000 frames, representing 40 trials each of 200 frames. In total then there would be 6 x 40 = 240 trials to track.

5. 1. Setting up batch tracking

Rather than load each video, press track, then save the tracking file, you can set Flote to batch track the whole experiment. Select Tracking→Setup Batch Tracking, to setup batch tracking.



First, press 'Source'. The 'Source' is the larger directory which contains all the video folders. Note that in the setup program, you can specify default locations for the source and target directories.

In the example above, you would select the folder 'Startle_05212006'. Press OK. The selected folder will appear in the box beside the **Source** button. Check that this is the one you meant to select. Flote will count the number of folders inside the selected folder. In the line below it should read
'Contains 6 folders, each with ...'

Next, you need to specify how many events/trials are inside each folder, and how many frames in each trial. Flote assumes that somewhere in the name of the folder is a string designating the experimental protocol in the form of "_Pxxx_" or "_Dxxx_" (where xxx are numerals). The first time you use a protocol, you will need to enter the number of trials and frames. However it will remember the settings for each protocol for future experiments. You can of course over-ride the remembered settings (which will then be replaced). If no string designating a protocol is found, or if the protocol hasn't been used before, Flote will guess that each trial is 120 or 100 frames long and enter these values accordingly. But this is likely wrong, you will probably need to enter these numbers yourself.

You must specify a directory where the tracking files will be saved. This can be done in either of two ways. Either press the **Target** button and choose a target folder. This will then be entered in the box beside the **Target** button. Alternatively, just click the **Auto** button. A new folder, with the same name as the folder containing the video stacks, will be automatically created in your 'save tracks' directory (see section 1 above).

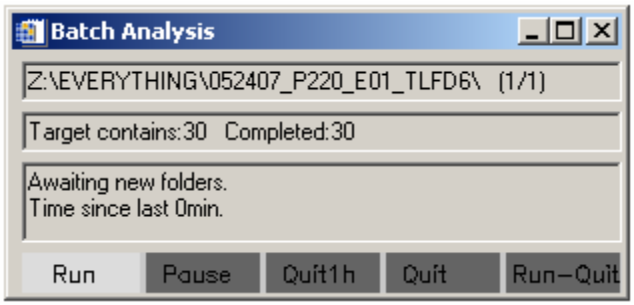
To have different experiments tracked with different configurations, choose a preset configuration from the drop-down list. Alternatively, select the first option '[User set]' to have the tracking not alter the selected settings in the main window. Note that if you have two experiments in the list where the first specifies a configuration file, and the second is at '[User set]', then the second will use the configuration loaded when the first experiment is tracked.

Finally, press 'Clear list' to remove any tasks that you may have specified in the past. Then click 'Add' to add this task to the bottom of the list or 'Add Top' to add this task to the top of the list. It should be displayed in the right-hand box, with the input directory, output directory, number of events and frames per event correctly specified.

If you performed more experiments you'd like to track, you can add these to the task list. Just don't click 'Clear list' in between - you can make a list of folders that should be processed. This is useful if you want to record all day, then let the tracking process everything overnight. When your list is complete, press 'Save List', then 'Quit'. If you press 'Quit' alone, the list will not be saved!

The 'Filter' field lets you specify which folders will be tracked. If blank, all folders are tracked, otherwise only folders containing the text in the 'filter' will be tracked. As tracking is done in an alphanumberic order of folders, this can be useful if you would like to see data from particular trials quickly.

5. 2. Running Batch Tracking



Make sure that your tracking parameters are correctly set. The same tracking parameters are applied to every experiment in the batch list. When you are ready to start, select Tracking→Start Batch Tracking. A batch tracking window will be displayed. This shows what trial is being processed and has several control options, selected by hovering the mouse above the field. Flote will not respond immediately to selection of these control options - if it is in the middle of a tracking operation, you need to wait until it is complete.

Batch tracking can be started while you are still recording videos. Once everything on the original list is finished, the source folders where you are saving video files are re-checked every 30 seconds to see if new video folders have appeared. If so these are then tracked. This is useful if you are recording on one computer and have another free to start tracking.

The batch control options are:
Run - continue batch tracking.
Quit1h - track until everything is finished. After 1 hour in which no new videos are saved in the source directories, quit.
Quit - Quit now.
RunQuit - track until everything is finished then quit.

If you have several computers networked, you can set multiple computers to analyze the same video files. Setup batch tracking on each computer, making sure that directory in which tracking data is saved is the same. You can then initiate batch tracking on multiple computers - each computer will first look to see if the relevant tracking file has already been saved. If so, it will skip to the next set of frames. For large experiments, having two or three computers analyze data can dramatically increase the speed of tracking. As a rough guide, analyzing an experiment with 100 folders, each containing 8000 frames (eg 20 trials of 400 frames) should take two computers working collaboratively about 10 hours.

In addition to the tracking files, the output folder contains a file 'log.txt'. This file, which can be opened by a word processor, contains information about the identity of the

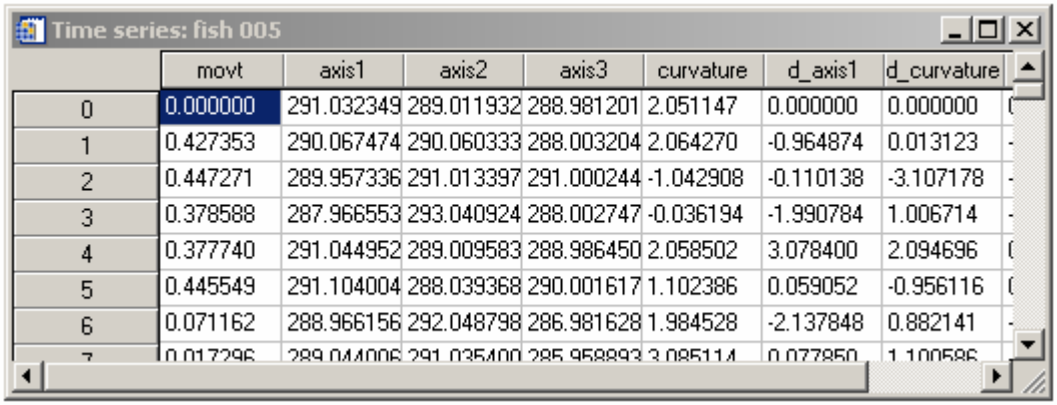
computer which performed the tracking, time and details of the configuration used. Each time the tracking started, the log file will be updated with this information. If multiple computers are used, they will all write to the log.txt file, allowing a quick check that the configurations were indeed the same. Network privileges may prevent some computers from writing to the logfile, in which case you will be notified of the error when tracking begins and the log file will not be updated.

To analyze the files created by batch tracking, see section 8.

6. Analyzing Time Series Data

After tracking the video, left click near the larva you are interested in. The larva should be designated by a circle around its head location (assuming you have Display→Selected Object→Highlight turned on). The dialog box will display two lines of information:
1. The Pixel location in (x,y) coordinates clicked and its intensity.
2. The number of the larva selected, its (x,y) coordinates and orientation in the frame selected.

A 'Time Series' window will open to the right of the main window. The time series window contains information on selected parameters for the highlighted larva. The value of each parameter across frames is shown. If this window is not desired, to prevent screen clutter, you can toggle its visibility using Windows→Hide Time Series.

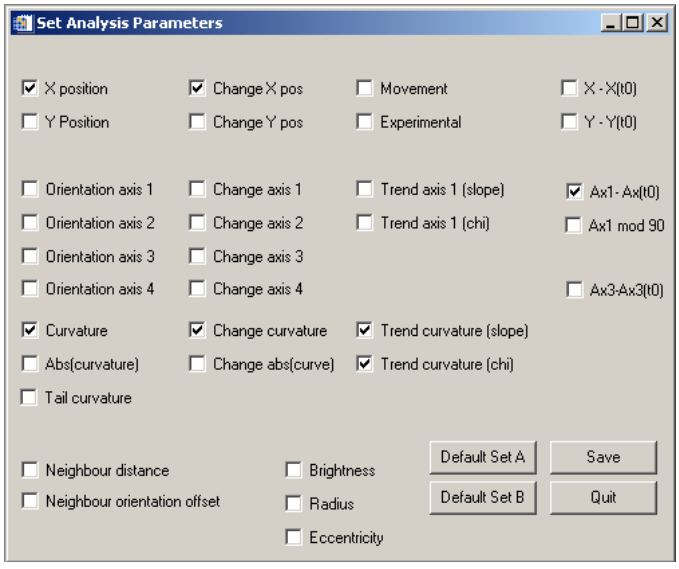


	movt	axis1	axis2	axis3	curvature	d_axis1	d_curvature
0	0.000000	291.032349	289.011932	288.981201	2.051147	0.000000	0.000000
1	0.427353	290.067474	290.060333	288.003204	2.064270	-0.964874	0.013123
2	0.447271	289.957336	291.013397	291.000244	-1.042908	-0.110138	-3.107178
3	0.378588	287.966553	293.040924	288.002747	-0.036194	-1.990784	1.006714
4	0.377740	291.044952	289.009583	288.986450	2.058502	3.078400	2.094696
5	0.445549	291.104004	288.039368	290.001617	1.102386	0.059052	-0.956116
6	0.071162	288.966156	292.048798	286.981628	1.984528	-2.137848	0.882141
7	0.017296	289.044006	291.035400	285.958893	3.085114	0.077850	1.100586

6. 1. Selecting time series data fields

A variety of parameters can be displayed in the columns of the Time Series window. Select which parameters are displayed by opening the Analysis→Select Params window.

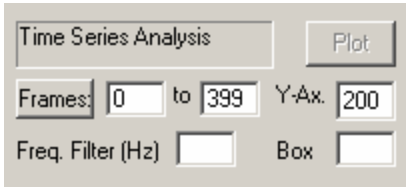
Click the checkboxes you want, then press **Save** and **Quit**. The **Default** buttons will mark two different subsets of the checkboxes that are typically useful. The checkboxes available are:



X Position - x coordinate of the object.
Y Position - y coordinate of the object.
Change X Pos - movement in x position from the previous frame.
Change Y Pos - movement in y position from the previous frame.
Movement - total movement in x,y position from the previous frame.
Experimental - not yet implemented.
X-X(t0) - x coordinate relative to initial position (i.e. in frame 0)
Y-Y(t0) - y coordinate relative to initial position (i.e. in frame 0)
Orientation axis 1-4 - orientation of body segments 1 to 4 respectively.
Change axis 1-4 - change in orientation of body segments from frame to frame (maximum change is 180 degrees, or 90 degrees when overlapping contours are used).
Trend axis 1 (slope) - slope of the linear fit to the head orientation function.
Trend axis 1 (chi) - chi-square error of the above function.
Ax1-Ax(t0) - head orientation relative to initial orientation.
Ax3-Ax3(t0) - tail orientation relative to initial orientation.
Ax1 mod 90 - head orientation converted to a 0-90 degree angle.
Curvature - sum of the angle between segment 1&2 and the angle between segment 2&3. A positive value means a leftward bend (be sure to check that your optics do not invert the image).
Change curvature - change in curvature from frame to frame
Trend curvature (slope) - slope of the linear fit to the curvature function
Trend curvature (chi) - chi-square error of the above function
Abs(curvature) - sum of the absolute value of the angle between segment 1&2 and the absolute value of the angle between segment 2&3
Change abs(curve) - change in the abs(curvature) from frame to frame.
Tail curvature - sum of the angle between segment 2&3 and the angle between segment 3&4
Neighbour distance - not yet implemented.
Neighbour orientation offset- not yet implemented.
Brightness - density of the head centroid
Radius - a measure of the size of the head centroid
Eccentricity - a measure of the symmetry of the head centroid

6. 2. Saving time series data

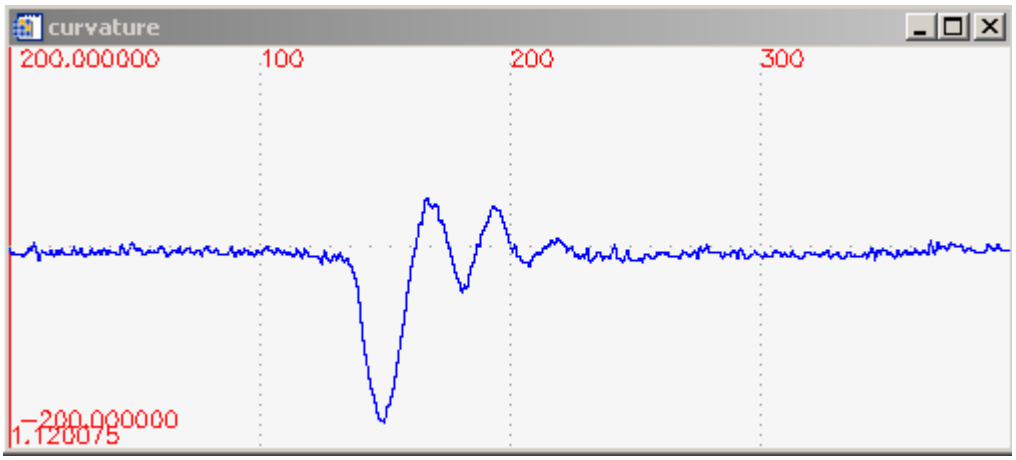
The values in the Time Series window can be saved by selecting Analysis→Save Time Series. The default file is 'C:\ftrack\singlefish.trk'. The output file is a tab separated text file that can be opened in excel. Note that if a file of the specified name exists, it will not be over-written, but the information appended to the end of the file.



Not all frames need to be saved. In the 'Times Series Analysis' area of the main window, the frames that will be saved are specified - the default is '0' to '399'. By changing these numbers, only the designated subset will be saved.

6. 3. Plotting time series data

To plot the time series, click on the column that you want to plot in the Time Series window then press the **Plot** button in the ' Times Series Analysis ' area of the main screen. A graph of the parameter against time will appear in a new window. The title of the graph is the parameter being analyzed. For example:



The vertical red line with the number at the bottom indicates the current frame and the value of the parameter plotted in the current frame.

Selecting a frame: Left clicking inside the Plot Window will change the frame displayed on the main image screen to the frame selected in the plot. To plot a different parameter, simply click on it in the Time Series Window. The red line in the plot window shows the frame selected and the value of the parameter selected at that time point.

Setting the Y-axis: To change the Y-axis of the graph, enter the desired value in the box 'Y-ax.'. If a single number is entered, the Y-axis is from -value to +value. If two numbers are entered (eg '0,200') the Y-axis will be set to those values. If you enter a blank in the box, the Y-axis will be automatically set, using the maximum and minimum values of the parameter respectively.

Setting the X-axis: To change which frames are plotted, enter new values in the 'Frames' fields in the ' Times Series Analysis' part of the main window. To set the x-axis to go from zero to the maximum frame number, click the 'Frames' button.

Calculating gradients: Holding the left button down and dragging within the Plot Window produces a light blue line showing the frame where the dragging commenced.

At the top of the light blue line is a bracket showing the number of frames and the difference between the parameter value at the beginning and end of the drag. The gradient of linear fit to the region between the blue line and the red line is calculated and shown as "m=". For convenience in copy-pasting into other documents, this value is also displayed in the message window.

As it is often useful to find the gradient for the same time window across larvae and framesets, the red-blue window is remembered when you click on new larvae or load new video sets.

Due to a quirk in the way IDL handles interactive windows, having a time series graph open usually interferes with attempts to select and open a new set of frames. While there is no problem click the 'Next' or 'Prev' buttons to get the adjacent frameset from the same folder, attempts to select a new folder for analysis using File→Select Video Directory will sometimes fail. If this is a problem, close the time series graph before opening a new folder.

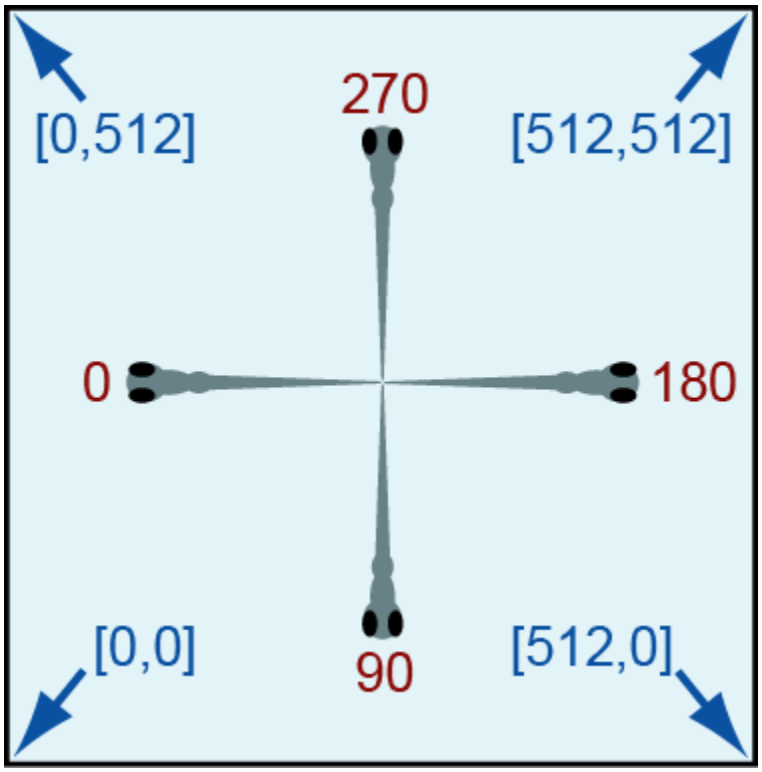
6. 3. 1. Smoothing time series data

There are three ways to smooth the time series data. Right clicking inside the Plot Window superimposes the Fourier smoothed trace (in black) with frequencies greater than 100 Hz removed. Right clicking a second time shows only the Fourier smoothed trace - in this condition the text at the bottom of the vertical red line shows the value of the Fourier smoothed trace. This is useful because the 100 Hz smoothed data set is used by Flote for determining movement episodes.

Entering a number in the 'Freq. Filter (Hz)' box allows for Fourier smoothing at the desired frequency. When anything is entered in the box, the zero frequency component is also removed. When a single number is entered in the box, everything above that frequency is also removed. When two numbers are entered separated by a comma, frequencies below the first number are removed, and frequencies above the second number are removed.

Entering a number in the 'Box' box performs box-car averaging of the trace, using a window of the number of frames entered in the box. For example, is 3 is entered, then the value at a time t becomes the average of t-1, t, t+1.

6. 4. Position and Orientation



Orientations in Flote are in degrees, with 0 being horizontally to the left. The x,y positions start at the bottom left of the screen. After tracking a larvae, left clicking on it brings up its number, centroid coordinates, orientation and orientation relative to a set point ('AVec') in the message window.

You can set the target point for the 'AVec' measurement by choosing Analysis→Misc Parameters and setting the x,y coordiates appropriately. This number is between -180º and +180º, with negative numbers meaning the target point is to the right of the fish and positive numbers meaning that it is to its left.

For example, after loading and tracking a video, you click near a larvae on the screen. The message window might now display the following text:

001 (163.1,232.9) Orient:263.3 AVec: -117.0

This means that you have clicked on larvae #1. Its x,y position in the current frame is x-163.1, y=232.9. Its absolute orientation is 263.3º (almost exactly upward), while its orientation relative to a target point (in this case [512,0], the lower right hand corner) is -117.0º.

7. Kinematic Analysis - Single Trials

Flote uses the raw data on position, orientation and body curvature to automatically classify the type of motor pattern represented by each movement event. This is the main purpose of Flote - to facilitate observer-independent, high throughput analysis of behavior.

An important warning - behavioral classification using kinematic analysis is *very* sensitive to the tracking and analysis parameters. Before embarking on large scale video recording, batch tracking and kinematic analysis, it is crucial to spend several hours manually loading videos into Flote, tracking and validating the behavioral classification. If this is optimized, you should be able to get attain a very low rate of mis-classification, less than 1 in 20, and those mis-classified events should be ones where the observer might also be uncertain.

Another key point is that because behavioral classification relies on the kinematic properties of movement, results obtained from analysis of mutant or drug manipulation need to be carefully analyzed. It is imporant to rule out that the effect is not a change in the kinematics of a movement such that it becomes mis-classified. For example, when analyzing the twitch twice mutant response to Dark Flash stimuli, we were surprised to see that they had a reduced frequency of O-Bend response and an increased frequency of J-Bend responses. However on careful inspection of the videos and kinematic data, it was clear that O-Bend kinematics are slowed down in *twt* such that the behavior now looks like a J-Bend. Thus for analysis purposes we aggregated O-Bend and J-Bend results.

Finally, while kinematic analysis and behavioral classification in Flote works well, the details of movement kinematics are not accurate for scoot maneuvers. This is because scoots (slow swims) are initiated with an extremely fine flexion of the distal tail, which Flote does not accurately measure. Thus Flote frequently identifies scoots only during the second peak of the sinusoid - so kinematic details of scoot movements are not reliable, although the classification as a scoot is still accurate.

7. 1. Setting Behavioral Thresholds

First, you need to setup the thresholds used for behavioral analysis. Select Analysis→Setup Behaviors. The fields are:

Analyze from frame - usually set to zero, meaning that frame 0 is the first frame analyzed. However you can set this to a larger number if you are not interested in movement events before this time.

Stimulus at frame - if there is a stimulus, you should enter the frame number when the stimulus began (this is an absolute number, not relative to the first frame used. So if the stimulus was at frame 210 and you decide to analyze from frame 200, you should set this value to 210, not 410).

MAX SLC limit (ms) - the window, in milliseconds, from the stimulus, during which movement events are considered SLC startle responses. Note that not all events initiated during this window will be classified as SLC responses, only those with sufficient bend angle and/or angular velocity. If no startle responses are expected, set this number to 0. Depending on temperature, this number is between 12-20 ms.

MAX LLC limit (ms) - the window, in milliseconds, from the stimulus, during which movement events are considered LLC startle responses. SLC responses are designated first, so if a response occurs within the SLC time limit, it will be classified as a SLC response. If a response occurs after the SLC time limit, but before the LLC time limit it will be an LLC response. Only responses which are larger than the scoot/turn threshold (below) are considered LLC responses. If no startle responses are expected, set this number to 0. Generally, this number is set at the size of the recording window.

Scoot/Turn thresh (deg.) - The maximum curvature for a scoot (generally 35 or 40 degrees depending on temperature) before it becomes a candidate for a turn/C-bend.

Turn/O-Bend thresh (deg.) - The maximum change in head angle for a routine turn before it is classified as an O-Bend (generally 95 degrees, depending on temperature).

Distance Integral (ms) - The frame interval for summing displacement to calculate the distance. For example if this is set to 1, the distance is calculated by summing the movement between frames for all frames. If it is set to 5, distance is calculated by summing the movement between every 5th frame.
Eyes - The number of eyes detected for the larva to be included in the analysis. This field should be set as [minimum number of eyes],[maximum number of eyes]. For example setting the field as "1,2" will include larvae detected as having one or two eyes as included. Generally anything with more than two eyes is not a larva and should be excluded from analysis. Larvae lying on their side are seen as having a single eye and it is often useful to exclude these by setting this parameter to '2,2'.

There are also several toggle switches under Parameters.

Ignore track errors - If a large jump in the head angle or body curvature occurs, this generally indicates a tracking error. Similarly, if a larva is very close to the edge of the recording field, a tracking error is generated. For startle analysis, we generally set this toggle on ; for swimming analysis we leave it off.

Ignore swimmers - The software attempts to detect larvae moving before the stimulus frame is reached. If this toggle switch is set, these larvae are indicated as 'swim'. If it is

not set and there is a distinct movement initiation before the stimulus frame, the movement will still be classified. We usually set this to on.

Ignore drifters - Flote detects larvae with an above threshold displacement before the triggers. In many cases these larvae are swimming and can be excluded using the 'ignore swimmers' toggle. However occasionally larvae are simply drifting in which case they can be excluded or included in the analysis using this toggle. If this toggle switch is set, these larvae are indicated as 'swim'.

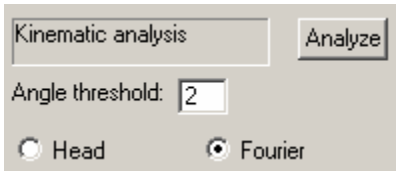
Strict SLC kinematics - If set, responses are only classified as SLC if they have a peak C1 angular velocity of greater than 18 and a C1 duration of less than 10. We usually leave this off.

Strict SLC latency - If set, responses are only classified as SLC if their latency falls within the 'MAX SLC limit' (specified above). We almost always set this to on.

After setting the parameters, save them, using File→Set. If this parameter set will be used frequently, then save it as a configuration, using Configs→Save New Config. In the dialog box, give the configuration a name and press the save button. Next time you open the Setup Behaviors window, your configuration should be present under the Configs menu. You still need to save it using File→Set before closing the window.

Finally close this window using File→Quit.

7. 2. Selecting Analysis Method



Next you should select an analysis method. In the 'Kinematic Analysis' area there are two options:

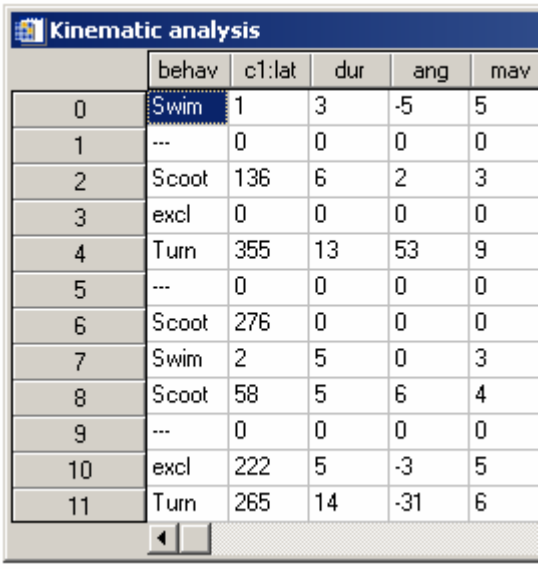
Head - uses the orientation of the head segment only to measure responses. This is suitable for measuring acoustic startle responses and is NOT appropriate for measuring scoot or routine turn movements.

Fourier - uses the curvature function to measure responses. This is much more sensitive and is suitable for measuring scoots and turns. It is not recommended for measuring startle responses.

Angle threshold - designates the sensitivity for picking up movements. For 'head analysis' this value means the change in head angle that must occur over a three millisecond window to identify a C-bend. This value defaults to 8, but can be set to a higher value for example 16 if spurious movements are being picked up. For 'Fourier analysis' this value refers to the derivative of the smoothed curvature function used to identify the beginning of a movement sinusoid and should be left at the default value of 2.

7. 3. Kinematic Analysis

Once you have set the behavioral thresholds and analysis method, click 'Analyze' in the 'Kinematic Analysis' part of the main window. The 'Kinematic analysis' window contains information about the kinematics and behavior of each larva. Each row represents a single larva, with the number corresponding to the number in the video. Clicking on a row will highlight the corresponding larvae and vice versa.



	behav	c1:lat	dur	ang	mav
0	Swim	1	3	-5	5
1	---	0	0	0	0
2	Scoot	136	6	2	3
3	excl	0	0	0	0
4	Turn	355	13	53	9
5	---	0	0	0	0
6	Scoot	276	0	0	0
7	Swim	2	5	0	3
8	Scoot	58	5	6	4
9	---	0	0	0	0
10	excl	222	5	-3	5
11	Turn	265	14	-31	6

Ignore for a moment the first column. The other columns (in order) give the details of the kinematics of movement bouts. The columns, in order represent:

c1lat - The time at which movement was initiated
dur - Duration of the first movement sinusoid
ang - Change in head orientation during the first movement sinusoid
mav - Maximal angular velocity achieved by the head in the first movement sinusoid
swvel - Swim velocity (pixels/time)
axis - Maximal curvature at the peak of the first movement sinusoid
c2dur - Duration of the second movement sinusoid
ang - Change in head orientation during the second movement sinusoid
mav - Maximal angular velocity achieved by the head in the second movement sinusoid
axis - Maximal curvature at the peak of the second movement sinusoid
displ - Straight-line displacement of the larva from the first to the last frame..
swcyc - Number of half swim cycles after the first two movement sinusoids (if this number is negative, it means the fish was still swimming at the end of the recording period. eg -5 means, there were 5 tail flips, but the fish was still swimming when the recording finished).
rhythm - Average duration of a swim half cycle
swmag - Average change in body curvature during a swim half cycle

swyaw - Average change in head orientation during a swim half cycle
t_end - Time at which movement ended
e_sw - Larva should be excluded from analysis because moving before stimulus.
e_oj - Larva should be excluded from analysis because orientation measurement error
e_ed - Larva should be excluded from analysis because too close to screen edge
e_tk - Larva should be excluded from analysis because position tracking error.
eyes - Number of eyes found anterior to the head centroid
traj - Trajectory of the larvae (angle moved at relative to initial orientation)
dist - The curved distance moved by the larva
frames - Number of frames for which the larva was tracked.

If you are seeing a lot of non-zero 'E-Oj' or 'E-tk' (more than 10% of responses) then you need to adjust your video recording or tracking parameters. Generally improving lighting and reducing extraneous contrast elements (i.e. dirt) will improve performance.

For angle and curvature measurements, the values are directional - negative values designate turns made in the opposite direction to positive values. In our system, negative values indicate rightward bends. However, as cameras and video recording software often reverse images, you should perform some tests to check whether this is true for your system as well.

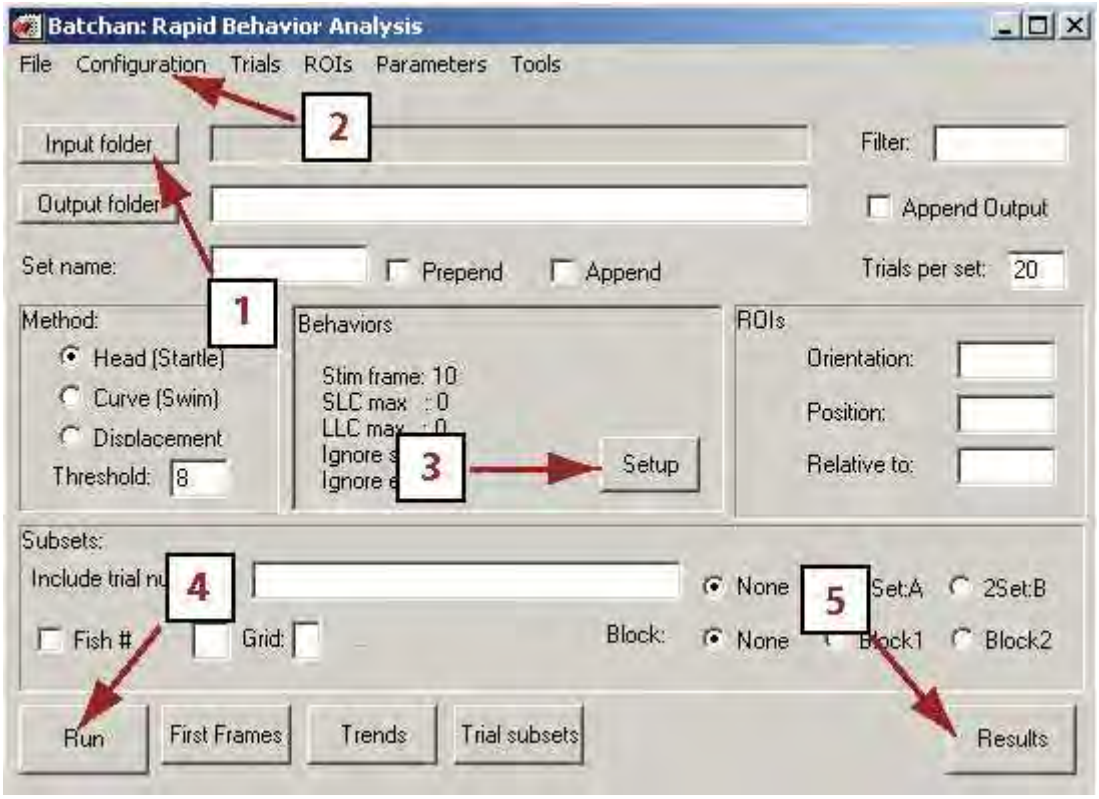
7. 4. Behavioral Classification

The first column specifies the type of motor pattern executed by the larvae in the video. This is based on the latency and kinematics of movement. The possibilities are

Swim - Larva initiated movement before the stimulus arrived (often the larva is already swimming at the beginning of the video).
SLC - A 'Short Latency C-bend'. These are Mauthner initiated startle responses, initiated at short latency to the stimulus.
LLC - A 'Long Latency C-bend'. These are non-Mauthner responses, initiated in a second wave after acoustic startle responses.
Turn - A routine turn.
TurnO - An O-bend.
Scoot - A forward swim.
Burst - A large amplitude forward swim.
J-Bend - A J-Bend (note this is not yet tested and should not be relied on).
Excl - Tracking error occurred (see below).
--- - No movement observed.

8. Batch Kinematic Analysis

After batch tracking the video files, you will have a directory of '.sav' files. These are then be analyzed in batch mode. You can now close the flote.sav program, and open the bathan.sav program.



The basic set of operations to analyze a folder of tracking files is as follows.

1. Select the folder contains the set of tracking files
2. Select a configuration from the pull-down menu
3. Open the behavioral classification menu by pressing 'Setup'
4. Run the batch analysis by pressing 'Single'
5. Analyze the results by pressing 'Results'

8. 1. Specifying Input/Output Files

Input Folder

Click the input folder button to select the folder containing the set of 'track_***.sav' files for analysis. After you have selected the folder, it should appear in the box beside the button.

Output Folder

Either:
(1) Click the output folder button and select a directory where the analysis files will be saved.
(2) Enter the name of a folder in the box beside the button. A directory of this name will be created inside the 'input folder' directory. If a directory of this name already exists, the analysis files inside will be over-written. The name you enter here is purely descriptive and has *no* influence on how the analysis is performed.
(3) Leave it blank and a name will be automatically assigned.

Filter

Only tracking files in the input folder whose name contains the text in 'filter' will be included in analysis. By default this is blank, so that all files in the folder are used.

Append Output

By default this is not checked and a new sub-directory of the name specified is created in the 'input folder' (as described above). If a directory already exists, the files in it will be over-written.

If this box is checked, then files in the output folder are not over-written, rather the new analysis is added to the end of the existing files.

Set name:

Normally this is left blank. The name of each set is then simply the name of the first file in that set.

For example, if a given group of fish (setA) was tested 30 times, then there will be a list of files in the tracking input folder 'track_setA_e00.sav ... track_setA_e29.sav'. The set will be called 'track_setA_e00'.

When text is entered in this box, the name of the set is exactly as specified in this box.

When text is entered in this box and the 'Prepend' button is checked, the text is added to the start of the first file name to create the name of each set. Thus if the text is 'trp1' then the name of the set will be 'trp1_setA_e00'. Similarly, if the 'Append' button is checked, then the text is added to the end of the first file name, for example 'setA_e00_trp1'.

Several of the ROI functions will automatically modify this field.

Trials per set

This number means how many tracking files will be averaged to form a single line in the c1_analysis.trk output file. This number generally specifies how many trials each group was subjected to.

For example, if a given group of fish was tested over 30 trials, you would normally enter 30 in this box. The percentage of fish responding with various types of motor pattern is then calculated as an aggregate of all trials. So if across 30 trials, there were 500 analyzable larvae identified and 78 O-Bend events, the %O-Bend=15.6.

However, note that this number can be used creatively to perform different sorts of analysis. In the example above, if you set the number to 1, then each line in the output file will represent a single trial. You can then determine whether there is a change in behavior over time (habituation or adaptation). Alternatively, you could set the number to 15, in which case each set of larvae will have two lines in the output file, the first representing trials 0-14 and the second 15-29, allowing a blockwise analysis to be performed.

8. 2. Setup Analysis

Setup the behavioral thresholds by clicking the 'setup' button in the middle of the window. Thresholds are set as in section 7.1 above. The 'Behaviors' box lists the current configuration.

Select an analysis method (head/swim/displace) and set the threshold as for section 7.2 above.

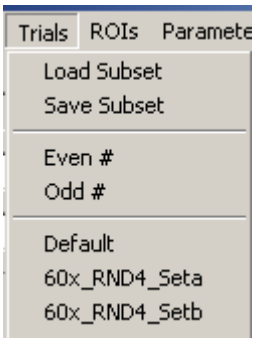
8. 3. Specifying Trial Subsets and ROIs

8. 3. 1. Trial Numbers

Often you will only want to analyze a subset of trials in an experiment. For example, you may have recorded 40 trials for each group of fish, but 20 trials were with a control stimulus and 20 trials with a test stimulus, in a pseudorandom sequence. Obviously, you then need to separately aggregate scores for those blocks.

To selectively analyze only certain trials, enter the list of trial numbers you wish to analyze. In the example to the right, only trials 1,2,4,8 etc will be analyzed. These correspond to tracking files ending in '_e01.sav', '_e02.sav', '_e04.sav', '_e08.sav' etc.

8. 3. 2. Trial Presets



If there are certain list of trial numbers you use frequently, you can save them using the Trials menu. When a list of trials is entered in the 'Include trial numbers' box, go to Trials->Save Subset, enter a name at the prompt. Trial lists are stored in the 'C:\ftrack\subset_configs' folder. If you ever wish to delete items from the Trial menu, open the 'C:\ftrack\subset_configs' folder and delete the correspinding file.

In the future, to use that set of trials, just open the Trials menu and select the set from the list.

It is advantageous to give trials subsets which are used in the same experiment, names which are alphabetically ordered, as it will later allow you to automatically cycle through them (see section XXXX below).

You can automatically generate the list of trial numbers when analyzing only even or odd numbered trials. If you have 40 trials, 20 of which are even numbered, then enter 20 in the 'Trials per set' box, then select 'Even #' from the 'Trials' menu.

8. 3. 3. Preset pseudorandom sequence

To the right of the trial numbers box are three checkboxes.

None - clears the sequence in the trial numbers box and also clears whatever name is entered in the 'output folder' field.

The other two boxes are for quickly entering a preset pseudorandom sequence, typically useful if there are 40 recording windows, divided into 2 groups (eg control and stimulus), which are intermingled according to the sequence set by these checkboxes. Activating the checkbox will enter a 20 number long pseudorandom sequence in the trial numbers box and name the output folder.

8. 3. 4. Blocks

If you only want to analyze a the first half of a sequence of trial numbers, click 'Block1' AND alter the number of trials per set to half the total number entered in the sequence

box. Similarly to analyze the second half of a sequence, click 'Block2'. This saves having the manually edit the trial numbers box.

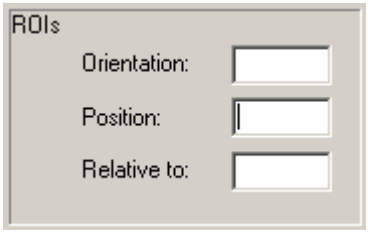
8. 3. 5. Gridded larvae

In some experiments you may have individual larvae placed in separate wells of a grid, and wish to analyze them separately. Flote can analyze larvae in different sized square grids, with the first larvae being in the bottom left corner. Be careful if the images loaded into Flote are flipped relative to the 'real world'!

It is not actually necessary to track the larvae using the grid option (see section 3.4.). When running analysis with a grid, Flote will in turn examine each region of the grid. Thus a fish in section 8 of the grid will always be called "-f8" irrespective of its original number during tracking. If more than one fish is found in a grid section, then Flote will assume that the additional fish represent contrast elements misidentified as fish. If you have set the tracking parameters correctly, these should generally be eliminated because they do not have the correct number of eyes - you should be able to tweak the 'eye finding' parameters so that incorrect contrast elements have zero 'eyes'. But if Flote cannot eliminate additional fish in a grid element using this procedure, then it will generate an error for that grid element in that trial. To see if errors are being generated in this way during analysis, turn on the Parameters→Report Track Errors option.

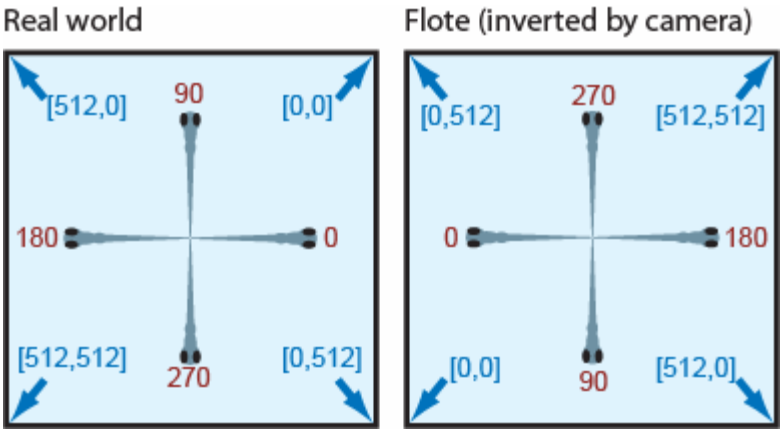
8. 3. 6. Regions of interest

In many cases it will be of interest to know whether larvae at a certain angle relative to a stimulus, or in a certain position within the testing arena behave differently from other larvae.



You can choose to analyze only larvae of a certain orientation by entering the range of orientations acceptable in the ROI box. The format is [mid-orientation, range] in degrees. So entering 90,45 means including only larvae in the orientation range 45 to 135. Similarly entering 0,10 would include only larvae whose orientation was in the ranges 355-360 and 0-5.

It is crucial to be sure of how your image of the testing arena is transformed during imaging. Most systems produce a 180 degree rotation by the time the image is loaded into Flote:



Orientations in Flote are anticlockwise, with 0 degrees horizontally to the left (in Flote, corresponding to horizontally to the right in the real world). However in some cases you may want to examine larvae of an orientation relative to a different angle, or relative to a specific point.

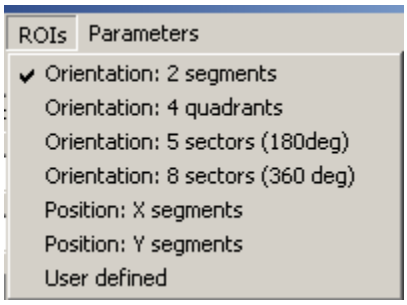
Orientation relative to an angle: Entering a single number in the 'relative to' box, makes the orientation ROI relative to that orientation. For example if you specify a range of orientations '90,20', then normally larvae whose orientation is between 70 and 110 would be included. But if you specified 'relative to' 45, then larvae between 115 and 155 would be included. Basically this just rotates the field so that the specified angle becomes zero.

*Orientation relative to point***:** Entering coordinates (eg '-100,256') in the 'relative to' box will include larvae whose orientation is in the range, relative to the angle from the position of the larva to the specified point.

You can also specific a region of interest in x,y coordinates in the 'position' box. The format is [start-x, end-x, start-y, end-y]. So for example entering '50,200,60,250' would only analyze larvae in a box starting from point (50,60) to point (200,250).

The number of larvae in a ROI can be found by looking in the xya_analysis.trk file in the 'AreaID' column. The percentage of larvae in the ROI is in the '%inArea' column.

8. 3. 7. ROI Presets



You can have Flote automatically analyze preset sets of regions of interest using the ROI dropbox. If you check one of the options in this dropbox Flote will do the following:

1. Enter a prefix for the ROI in the 'Set name' field.
2. Enter the corresponding ROI in the ROI box.
3. Run an analysis.
4. Check the 'Append output' box.

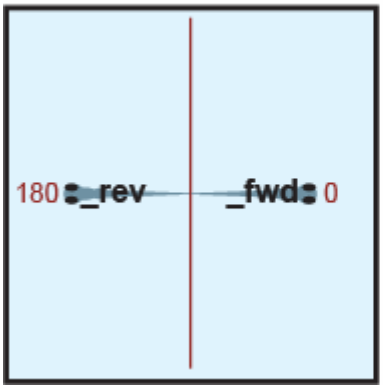
5. Return to step 1 and cycle through until all ROIs for the set are analyzed.

You will normally need to have clicked the 'Prepend' checkbox so that the prefix in the set name field is added to the beginning of the name, rather than become the entire name.

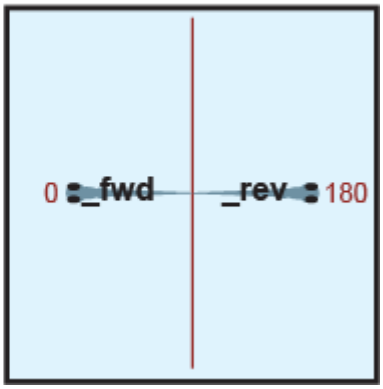
Preset ROIs are available, covering sets of orientations as well as regions of the testing arena. These are illustrated below. Keep in mind that the diagrams representing orienation groups may be confusing. For example, for the 'Orientation 4 quadrants' ROI the diagram does not indicate that '_twd' larvae are those on the left of the testing arena, it illustrates that '_twd' larvae are those fish *anywhere* in the arena, which have an orientation from within 45 degrees of 0.

Orientation: 2 segments

Real world



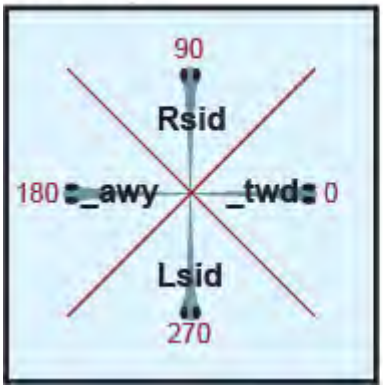
Flote (inverted by camera)



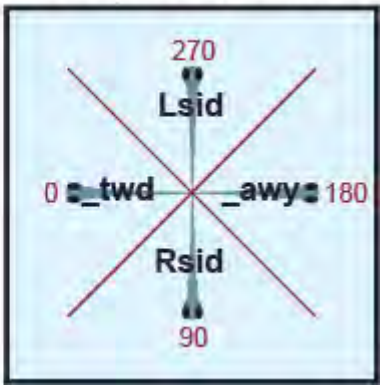

```
_fwd: orient 0,90
_rev: orient 180,90
```

Orientation: 4 quadrants

Real world



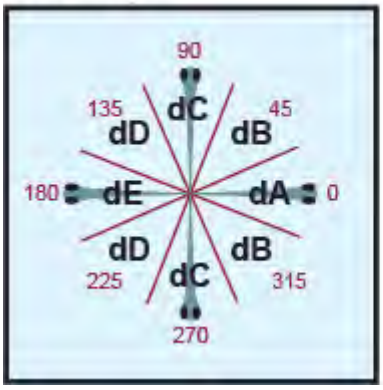
Flote (inverted by camera)



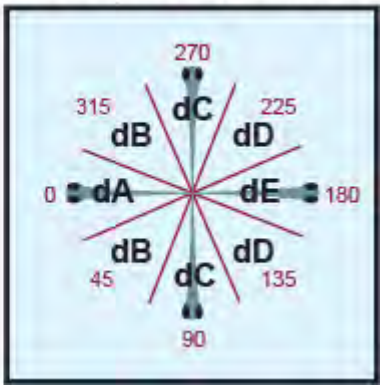

```
_twd: orient 0,45
Rsid: orient 90,45
Lsid: orient 270,45
_awy: orient 180,45
```

Orientation: 5 sectors

Real world



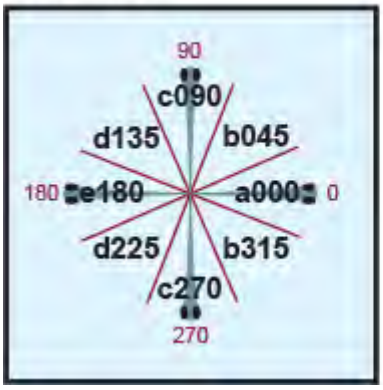
Flote (inverted by camera)



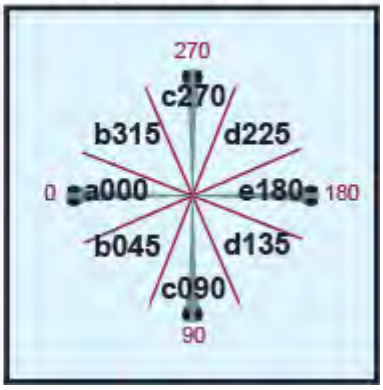

```
dA: orient 0,22
dB: orient 45,23
dC: orient 90,22
dD: orient 135,23
dE: orient 180,22
dD: orient 225,23
dC: orient 270,22
dB: orient 315,23
```

Orientation 8 sectors

Real world



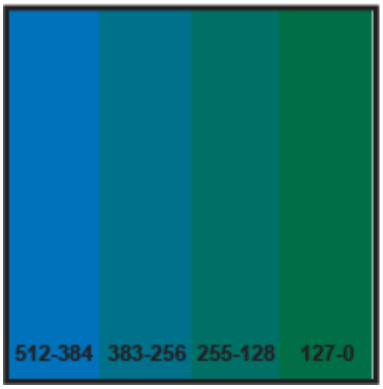
Flote (inverted by camera)



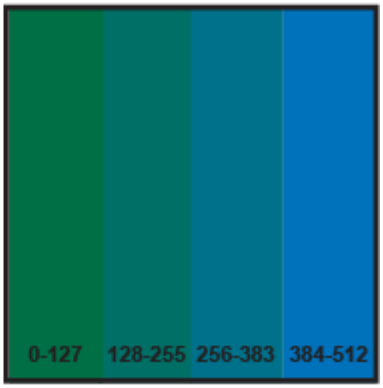

```
dA000: orients 0,22
dB045: orients 45,23
dC090: orients 90,22
dD135: orients 135,23
dE180: orients 180,22
dD225: orients 225,23
dC270: orients 270,22
dB315: orients 315,23
```

Position: X segments

Real world



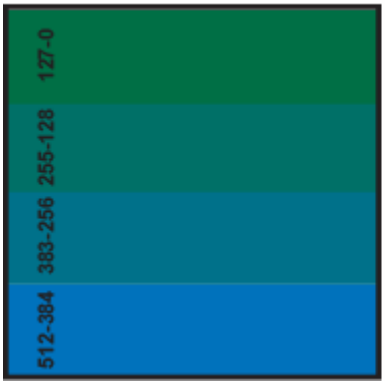
Flote (inverted by camera)



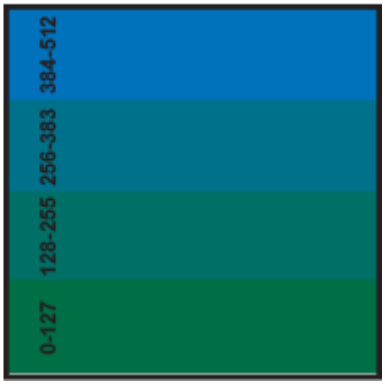

```
Xseg1: 0,127,0,512
Xseg2: 128,255,0,512
Xseg3: 256,383,0,512
Xseg4: 384,511,0,512
```

Position: Y segments

Real world



Flote (inverted by camera)

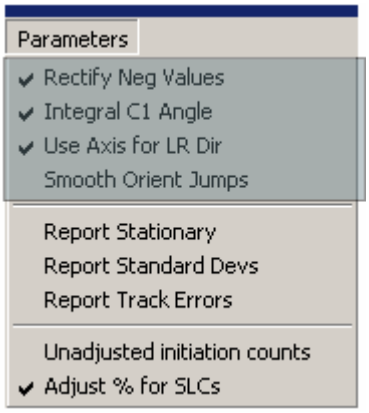



```
Yseg1: 0,512,0,127
Yseg2: 0,512,128,255
Yseg3: 0,512,256,383
Yseg4: 0,512,384,511
```

If you select the 'User defined' option, Flote will open the file c:\ftrack\paths\userroi.txt. This file should be organized as series of lines where each line specifies a the name of an ROI and its parameters: *Name;Orientation ROI;Position ROI*. All these elements must be present. For example to analyze first the 90 degree angle centered on orientation 0, then the 90 degree angle centered on orientation 180, over the entire recording field, the file could contain these two lines:
Sect1;0,45;0,512,0,512
Sect2;180,45,0,512,0,512

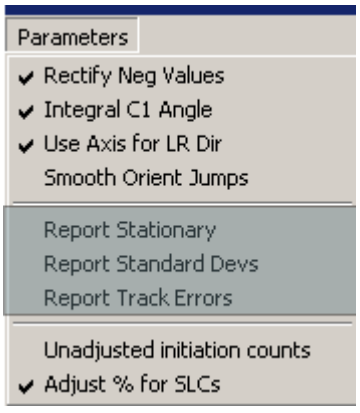
8. 4. Setting options

8. 4. 1. Analysis Parameters



When saving kinematic data to the summary.trk file, Flote can either save changes in orientation and curvature as values greater than zero (ie the absolute magnitude), or as values greater than zero for leftward turns, and less than zero for rightward turns. Toggle the Parameters→Rectify Neg Values ON to save only the absolute magnitudes.

8. 4. 2. Reporting Options



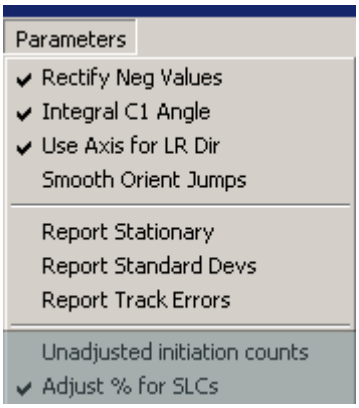
1. Report Stationary - By default, Flote does not include information about stationary larvae in the sumary.trk file because this makes the file very large and both batch analysis and subsequent results analysis can become extremely slow due to the large amount of data being manipulated. However, information on larvae that are deemed to be 'stationary' can be necessary when performing displacement analysis, so toggle this on to get information about the x,y coordinates, orientation and density of stationary larvae.

2. Report Standard Deviations - Output cells contain standard deviations instead of mean values.

3. Report File Errors - Track files are sometimes be corrupted, unable to be opened or containing scrambled data. Flote will simply skip these files, however, when checked, this option pops up a window detailing which files were corrupted. You should go back and retrack these files (just delete the tracking files affected and initiate tracking again - Flote will skip over trials where a tracking file exists and just track trials where there is

no corresponding tracking file). However occasionally you will want to ignore the scrambled files and turn off error reporting - uncheck this option to do so.

8. 4. 3. Calculation Options



There are three ways that initiation frequences can be reported.

1. The default way is to simply report the percentage of maneuvers of a given type (ie SLC, LLC, O-Bend etc). This represents the fraction of these maneuvers observed out of all analyzable embryos spotted.

2. 'Unadjusted initiation counts' - toggle this on to get the 'raw' number of maneuvers observed, ie NOT divided by the number of analyzable embryos. This is mostly useful when estimating directional bias - you may decide to exclude items where too few maneuvers were observed to give reliable data.

3. Adjust % for SLCs. Normally, the number of LLCs is reported as the fraction of larvae executing a LLC out of all analyzable larvae that did not perform an SLC. Remove this toggle to get the number of LLCs as a fraction of analyzable larvae.

8. 5. Loading and saving configurations

<To be written>

8. 6. Output files

The sub-directory created by batch analysis contains four output files plus a 'config.txt' file which contains a record of the settings used for the analysis.

1. summary.trk - Contains kinematic data on the first two bends (eg C-Bend and counter-bend) for each movement episode measured. This is a tab-separated text file that can be opened in excel.
2. swim_summary.trk
3. c1_analysis.trk
4. xya_analysis.trk

These files can be opened in a spreadsheet (Excel) or used as input for the kinematic analysis

A quick overview of the contents of each of the output files

summary.trk

This file contains information on kinematic properties of the larvae during the first two peaks of the waveform of movement. For example for startle responses, this file contains information about the C-bend and Counterbend.

	A	B	C	D	E	F
1	anadata8b: Response Analysi	Date:Mon Jul 23 11:30:47 2007				
2	File	Fish	Resp	Xpos	Ypos	Orient
3	dA_i0000p3_00a_e03_0000	2	2	151.03	127.182	335.022
4	dA_i0000p3_00a_e03_0001	6	2	330.054	223.738	340.032
5	dA_i0000p3_00a_e03_0003	10	2	374.347	296.028	21.0553
6	dA_i0000p3_00a_e03_0005	3	3	109.051	130.864	315.054
7	dA_i0000p3_00a_e03_0006	4	3	87.9844	144.805	320.006
8	dA_i0000p3_00b_e03_0000	7	3	26.1097	250.957	359.992
9	dA_i0000p3_00b_e03_0000	11	3	261.078	327.32	21.9057
10	dA_i0000p3_00b_e03_0001	6	2	95.3231	246.151	3.93429
11	dA_i0000p3_00b_e03_0001	8	2	23.1483	282.971	32.0214

Column A specifies the video sequence in which the event occurred. Here, a prefix was used so the file name begins 'dA_' instead of 'track_'. Thus first event was in the folder 'i0000p3_00a'. The next section '_e03' means that during analysis, a subset of videos must have been selected, starting with the fourth video sequence in the whole stack. More usually it would read '_e00' meaning that analysis began with the first video in the stack. Finally, '_0000' refers to the number of the video within the subset selected. In this example, analysis began with the fourth stack, thus _0000 also the fourth stack. The next line is '_0001', which would refer to the fifth video in the stack.

Column B specifies the identity of the fish in the video. Once you've identified the video sequence and fish, you can return to Flote and watch the movement of that particular fish. As an additional check, compare the initial (x,y) coordinates and orientation of the fish (columns D,E and F).

swim_summary.trk

This file contains information on kinematic properties of the larvae during swimming.

c1_analysis.trk

This file contains information on the behavioral classification of the larvae.

File - the name of the first file in the set of trials combined for analysis.

TotIDd - total number of fish identified over all the trials

E-Ed - the percentage of fish excluded from analysis due to being too close to the edge of the image.

E-Jmp - percentage excluded because orientation analysis was unreliable (usually poor image quality)

E-Trk - percentage excluded because due to a tracking error

E-Sw - percentage excluded because larvae was already moving before the stimulus time

E-Eye - percentage excluded because the object did not have the specified number of eyes in the first frame of the recording. Note that this can be a very useful measurement, as if you specify that the eyes should be '2,2' and are confident that there are no odd contrast elements in the video, then you have a measure of the number of larvae that are not perfectly upright. This is thus a nice surrogate for vestibular function.

TotAnd - total number of fish analyzed after excluding due to errors

React - percentage of fish performing any response (i.e. not stationary)

SLC - percentage of fish performing an SLC

LLC - percentage of fish performing an LLC

AllTurns - percentage of fish performing an O-Bend, R-Turn or J-Bend

R-Turn - percentage of fish performing an R-Turn

O-Bend - percentage of fish performing an O-Bend

J-Bend - percentage of fish performing a J-Bend

Swim - percentage of fish performing a Scoot or Burst

Scoot - percentage of fish performing a Scoot (slow swim)

Burst - percentage of fish performing a Burst swim

Other - percentage of fish performing a response not identifiable by Flote

SLC(r) - the percentage of SLCs initiated in a rightward direction

LLC(r) - the percentage of LLCs initiated in a rightward direction

AllTurns(r) - the percentage of (Obends+RTurns+JBends) initiated in a rightward direction

RTurn(r) - the percentage of R-Turns initiated in a rightward direction

OBend(r) - the percentage of O-Bends initiated in a rightward direction

JBend(r) - the percentage of J-Bends initiated in a rightward direction

Sct(r) - the percentage of Scoots initiated in a rightward direction (note - this is NOT reliable, it is included for future improvements)

Bur(r) - the percentage of Bursts initiated in a rightward direction

BiasSLC - the tendency for SLCs to be initiated in the direction of the target. For this to be useful, you need to specific the position of the target (see section 8.3.6). This is calculated at 2*[%SLCs initiated in the direction of the target]-100. Thus a score of +100 means all are target directed, -100 means all are away from the target.

BiasLLC - the tendency for LLCs to be initiated in the direction of the target

BiasTurn - the tendency for (OBends+RTurns+JBends) to be initiated in the direction of the target

BiasRTurn - the tendency for R-Turns to be initiated in the direction of the target

BiasOBend - the tendency for O-Bends to be initiated in the direction of the target

xya_analysis.trk

This file contains the information that can be used to make simple analysis of distance, speed and orientation of the larvae.

File - the name of the first file in the set of trials combined for analysis.

TotIDd - total number of fish identified over all the trials

TotAnd - total number of fish analyzed after excluding due to errors

AreaID - total number of fish analyzed that are in the ROI and/or Sector

%inArea - percentage of all fish that are in the ROI and/or Sector

XVec - mean horizontal displacement from center of all larvae identified where -1 and +1 represent the extreme edges of the recording field.

YVec - mean vertical displacement from center of all larvae identified where -1 and +1 represent the extreme edges of the recording field.

AVec - mean orientation of the larvae relative to a specified orientation or target position.

T-Dist - mean distance of larvae from the target position

AVecL -
AVecR -
XVecS -
YVecS -
AVecS -
SwVec -

9. Results Files

10. Results Analysis

Flote contains a simple utility to quickly graph and assess the significance of difference between experimental groups.

Please note - this utility is for a quick analysis *only*!!! Flote does not give you the flexibility to appropriately set up or validate the statistical tests used. You ***must*** import the data into an appropriate statistical package like SAS or SPSS.

10. 1. Loading Data

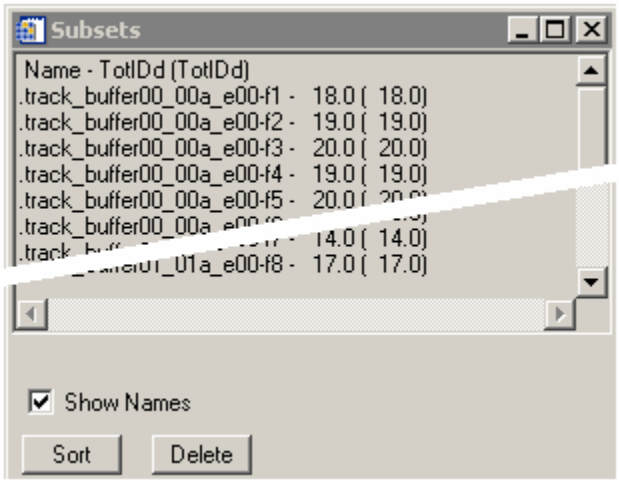
Upon entering the statistical package, you are prompted for a file to load. Select any '.trk' file generating by the analysis package. At any time you can load a new file by selecting File→Load

The currently loaded file is shown just below the menu bar. At any time this file can be refreshed from the hard drive by selecting File→Reload File

In some instances you may have multiple experiments with the same

10. 1. 1. Window Controls

On selecting File→Quit all windows will be closed *except* for the **Printer Out** window. This will remain so that you can add data to the printer output window from other analyses.



10. 1. 2. Groups Window

This window shows the groups that match the selected set together with the value of the parameter selected.

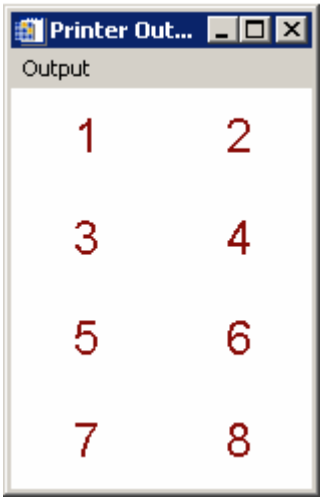
If no sets are selected then all groups are displayed.

If a range of values has been selected, then the display is sorted so that groups inside the range of values are shown, with a '*' mark in front of their name to show that they fall within the selected range.

If the range of values was selected for a parameter other than the one currently being displayed in the graph, then the brackets on the right show the value for the parameter for which the range was selected.

The **Sort** button will reorganize the list alphabetically. The **Delete** button lets you eliminate a group from further analysis (see below). The **Show Names** checkbox (not implemented fully) by default is on, allowing you to see the group name and value. When off, it just lists the values for the selected group to facilitate cut/paste into statistical packages.

10. 1. 3. Printer Out



If you click on this window with the left mouse button, the contents of the main window will be copied to the printer out at that position of a 2 row by 4 column grid. If you click on the printer out window with the right mouse button, the contents of the main window will be copied at that position of a 4 row by 8 column grid.

To send the contents of this window to the printer, select Output→Print from the menu at the top. Similarly, the output window can be saved as a '.tif' file or copied to the clipboard from this menu. To clear the contents of the window, select Output→Clear.

10. 2. Define sets

The analysis package is built to allow you to easily clump a set of 'groups' (which may be plates, single fish or individual movement responses) into a 'set'.

Sets are defined on the basis of having a common sequence of alphanumeric characters in their name. Thus if your have a series of groups called
A_con_00a
A_con_00b
A_con_00c
A_met_00a

A_met_00b
A_met_00c
B_con_00a
B_con_00b
B_con_00c

Go to Define Sets and select the number of characters from the end you wish to ignore when matching names. For example, if you select '-1' then the first 8 characters will be used to define the group identity. In the example above you would end up with 3 sets: A_con_00, B_met_00 and B_con_00, each of which includes 3 groups (the first set would be A_con_00a, A_con_00b, A_con_00c). Alternateively use Define Sets→Enter End to open window with the first group name. Click within the group name to show how many characters from the beginning should be matched.

Now, the 'sets' droplist will contain just the sets that have been defined in this way. If you select a given set, the matching groups will appear in the 'subsets' window.

Important. Before defining the sets, you should remove groups that you want to exclude and setup parameters that help you to define appropriate sets - see below. Strange things can happen if you don't define the sets first.

10. 2. 1. Remove unwanted groups

Before defining sets, remove any groups that you want to discard from further analysis.

To delete a single group, in the subset window, select a group to discard and press the delete button.

To delete groups whose values lie outside a range, use the histogram window to select a range of values for a given parameter, by holding the mouse button down and dragging across the range you want, then press the 'cut' button.

10. 2. 2. Parameters to identify sets

Under 'Define Sets' a number of options provide flexibility in identifying sets.

10. 2. 2. 1. Single Fish

This should be checked if you have an experiment in which each group is actually a single fish so that the name of each group contains a string '-f1' (for fish 1) to -'f9' (for fish 9). If this is checked, Flote will automatically discard the '-fX' information in attempting to make sets. If you do not have single fish, its OK to leave this checked because if Flote can't find the string '-fX' then it will ignore this option.

For example, if you have eight groups: 'Con' and 'Exp', each containing 4 fish, then your group names will be 'Con_e00-f1', 'Con_e00-f2', 'Con_e00-f4' etc. By selecting this option, Flote will ignore the '-fX' so that you can easily define two sets 'Con' and 'Exp'

10. 2. 2. 2. Smart Names

When this is checked, Flote will attempt to guess the names of the sets. In doing so, it assumes that each group name begins with the string 'track_' and it will remove this string from the group names.

Smart names is automatically turned off in several cases
1. If you used the 'prefix' option when running the analysis, then the word 'track_' will not be present and Flote will turns **smart names** off
2. If you are analyzing the summary.trk file or other data where the group name does not end in '-fX' (for fishwise analysis) or '_eXX', then Flote turns **smart names** off.

This option can be a source of grief - if you find weird things happening, like groups being ignored, or datapoints lost, the first thing to try is turning this off. 'Smart names' is not so clever.

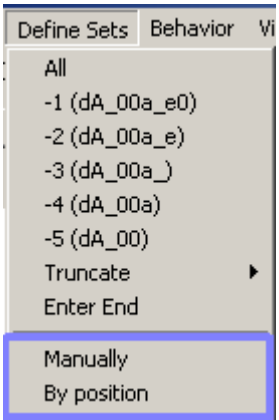
10. 2. 2. 3. Ignore Case

Self-explanatory

10. 2. 2. 4. First +1

Sometimes you want Flote to define sets, ignoring the first character in the string. An example: you analyzed the data according to quadrants, and now you have a 'Rsid_' group and a 'Lsid_' group. For the purpose of analyzing the rightward bias of turns, its desirable to analyze Rsid and Lsid separately, so you would define the groups as normal. But for the purpose of analyzing the turn-bias toward the target, or movement kinematics for side-oriented larvae, it is better to have the Rsid and Lsid measurements in the same set. So you can check the **First+1** option and take the average of the Rsid and Lsid measurements.

10. 2. 2. 5. Matching groups



If you enter a string in the 'match' box, then only groups matching that string will be included in the sets. Others are simply ignored.

10. 2. 3. Other ways to identify sets

The Define Sets menu has two special modes for defining sets.

Sets can be defined 'manually'. Selecting this option brings up a window asking for a string to match 'Set 1' with. Any group which matches the text you enter here will be part of 'Set 1'. Continue entering strings that you want to match each set with. When you are finished, leave the box blank and hit 'OK'. This option also allows you to give the sets a name, by entering the match string, then a comma, then the name you want the set to have. For example entering 'a01a,First' will match all groups with the string 'a01a', and the set will be called 'First' (in the bar graph mode).

Sets can be defined 'By position'. This is useful when larvae are in individual wells in a 3x3 grid and were analyzed with the grid option. All the 'f1' larvae will be a set, the 'f2' larvae another set and so on. This is mostly intended to allow quick verification that position in the array is not influencing behavior.

10. 3. Changing the Plot Window Format

Right click inside the plot window to set the title of the window. To clear the title, right click and leave the box empty.

To change the color scheme use the controls in the Format menu. You can individually set the:
Background - color of the blank area behind the plot
Lines - color of the plot axes and text
Sets - color used to plot the value of all the sets
Selected - color used to plot the value of the selected set
Subset - color used for plotting when a sub-range of values has been specified.

Six preset color schemes can be quickly specified. These are the options at the top of the Format menu. The schemes are described as Sets/Selected/Subset. Selecting White/White/White automatically turns on the

Text size: The format menu also allows you to change the size of the font used (Format→Large Text or Small Text). The large text option is particularly useful when plots are being added to the printer output window in half size. The font can also be changed to a pseudo-Helvetica format for a chic Swiss look.

Thick lines: The weight of the lines can be changed to a thicker appearance, using Format→Thick Lines, also mostly useful when plots are being added to the printer window in half size.

Outline bars: In graphs where the data appears as bars (histogram, bar graph, box/whiskers) selecting Format→Outline will circumscribe each bar with a black line (or whatever color you selected for *Lines* above).

Grid: Horizontal grid lines can be added to the plot by selecting Format→Grid.

Names vertical: In bar graph mode, if there are many bars, the names of the bars often do not fit underneath them. Names can be instead placed in a vertical direction on the left side of the bar by selecting Format→Names vertical.

Error bars: In graph modes with error bars, the bars are plotted only upward by default. To have errors bars also stretching downward select Format→Bidir err.

The size of the plot area can be changed by dragging the edges. To restore to the default, select Format→Restore. To grow or shrink the plot area by standard amounts select Format→Shrink or Enlarge. Similarly Show→Resize has some options for controlling size.

Set the X and Y axes using Format→Set X-Axis and Format→Set Y-Axis. To restore the axes to automatically scaling, enter nothing in the dialog box. A shortcut to restore axes to auto-scaling is to click anywhere in the area to the left of the Y-axis.

10. 4. Histogram Plots

In histogram mode, datapoints are binned according to user-specified bin dimensions. The X-Axis shows each bin, displaying the high-value for datapoints in that bin.

Set the bins of the histogram using the three boxes 'Min', 'Size' and 'Bins'. In addition, a first bin and a last bin will be automatically generated for datapoints lying outside the specified set of bins.

Min - the highest value for the first bin. This will include all data points less than or equal to this value. For example if set to 10, the first bin will include all data points less than or equal to 10.
Size - the width of each bin (except for the first and last). For example if set to 20, then in the example above, the second bin will include all data points greater than 10 and less than or equal to 30.
Bins - the number of bins between the first and last bins. Thus if this is set to 10, then in the above example, the 12th bin will be for datapoints greater than 210.

10. 4. 1. Selecting Bins

To select a group of bins, hold the left button and drag the mouse across the columns you want to be selected. To select everything less than a given bin, release the mouse button beside the left-hand Y-axis. To select everything greater than a given bin, release the mouse button beside the right-hand Y-axis. On the top right of the screen will be displayed 'where ...' indicating the subset selected.

10. 4. 2. Comparing Sets

By default, the distribution of a given set is superimposed on the overall distribution of all data points loaded. To examine a set in isolation uncheck the 'Background' checkbox.

10. 4. 3. View Presets

The four buttons on the left are available to set as preset views, including the parameter and bin setup. A keyboard shortcut for these buttons is available: press 1,2,3 or 4 to select the corresponding bit.

To set the views use the Views menu - the selection will be set to match the current configuration.

10. 5. Data points

This plot shows the individual group values for each set, stacked vertically. Dots are randomly displaced in a horizontal direction to make it easier to see and avoid dots overlapping. Click in the graph to redistribute horizontal positions.

10. 6. Bar Plots

Bars are displayed in the order that the sets appear in the Sets droplist. To change this order, select a set and use the **L** and **R** buttons to move the set up and down (=left and right in the bar graph) in the droplist.

10. 6. 1. T-tests between sets

To compare all other sets to a selected sets, toggle ON the Show→T-test option. A shortcut is to double click on a bar - this will select that set, and toggle the t-test option on. Single click on another set to make it the comparison set. To clear the comparisons toggle OFF the Show→T-test option, or for a shortcut, click just to the right of the left Y-axis.

To compare two sets, hold the left mouse button down above the first bar and drag across to the second bar then release the mouse button. You can do this multiple times. To clear the comparisons, hold the left mouse button while dragging the mouse from beside the left Y-axis to the right Y-axis.

All t-tests are two-tailed and assume equal variances.

By default, t-tests p values are directly given. However by toggling the Show→p values option, you can display symbols for different levels of significance.

10. 6. 2. Single factor t-tests

To compare the mean of a set to a specified value, select Show→One sample t. A dialog will open asking the numerical value for comparison.

10. 6. 3. T-tests between parameters

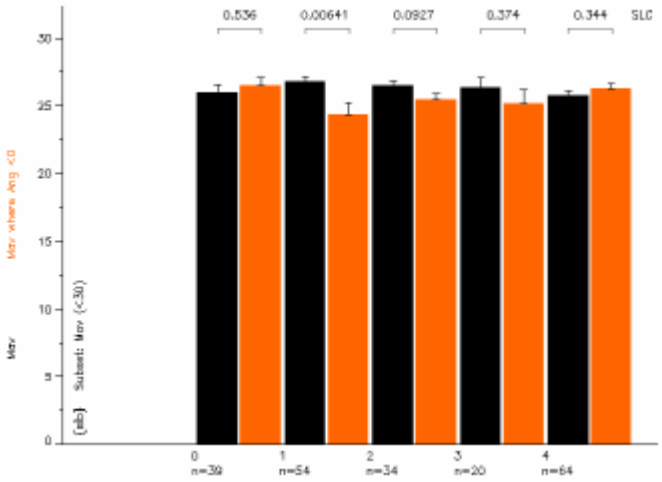
To compare the mean of a given parameter for a set, to the mean of another parameter for the same set, select a secondary parameter using the top right droplist. The two parameters will then be graphed side by side for each set.

10. 6. 4. T-tests within parameter, different range

This is accomplished by selecting a range of values for one parameter in histogram mode, then in bar graph mode, setting both the parameter and secondary parameter fields to be the same.

Best explained with an example. You want to compare the duration of C-bends for left directed turns and right-directed turns. So you performed analysis with the Parameters→Rectify Neg Values option OFF. You open the summary.trk file and after eliminating datapoints and defining sets as usual, you do the following:
1. In histogram mode, chose the Angle parameter
2. Select the range of values less than zero by holding the left mouse button down slightly to the right of the left Y-axis, then dragging across to the side of the bar in the zero value.
3. Go to the bar plot mode and chose the duration parameter - you should now have a simple bar plot showing the mean of the duration for each set.

4. In the top right corner, set the secondary parameter also to be 'duration' - the original bar will change size slightly because it now only shows the duration for angles greater than zero (and the n below each bar will only reflect the number of groups with angle greater than zero). In addition a new bar will appear beside it, showing the duration for angles less than zero. The label beside the right axis will reflect this.
5. Select Show→T-test to get the p value of comparisons between bars.



Note: for this to work properly you must use a 'standard size window' (Format->Restore).

10. 7. Trend Plots

10. 8. Bar Trend Plots

10. 9. Box Plots

The box marks the interquartile range (i.e. 25% to 75%) with the median as a black strip across the box. The whiskers represent the range of all points except for outliers, where outliers are points 1.5x the IQR below the first quartile, or above the third quartile. There is no current option to represent the outliers.

To see what the values for the whiskers, interquartile range and median are, select Show→Average/SD.

10. 10. Scatter Plot

10. 11. Circle Plot

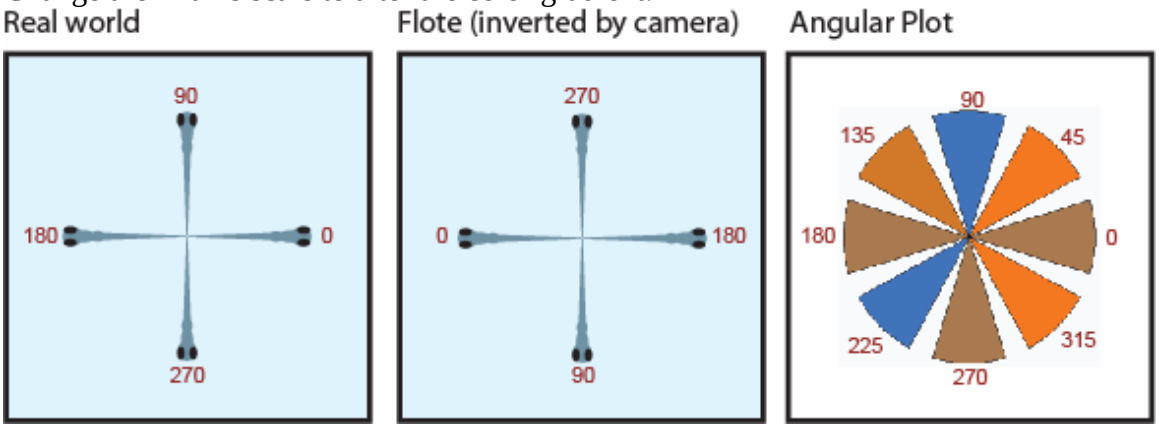
10. 12. Angular Plot

The angular plot takes the groups and plots them as sectors on a circle, evenly distributed. This is most useful as a visual aid to comparing behavior analyzed using the 8 sector region of interest mode. The color of the bar indicates the value, with the scale bar in the right hand corner showing the range of colors and corresponding values.

Use the Format→Thick Lines option to toggle the size of the bars.

Use the Show→Average/SD option to toggle the name and value of the bars.

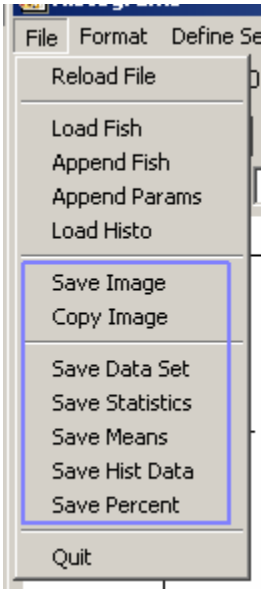
Change the Y-axis scale to alter the color gradient.



An advantage to using this method of analysis is that the 8 sectors now are visually displayed in the same orientation as the real world.

10. 13. Saving Data

The File menu offers several options for data output. There are two options for obtaining a copy of the graphical window:



Save Image - save window as a tif file

Copy Image - copy window to clipboard for pasting elsewhere

There are additional options for saving tab separated text files of the data set.

Save Data Set - save a tab separated (ie excel compatible) file with the current data loaded. This is an easy way to save data after deleting groups and data points. The file is found in the currently working folder, and has the same name as the currently loaded data set, with the string '_data' appended.

Save Statistics - save the current mean, standard deviation (or SEM,

whichever you are currently showing) for all currently defined groups as well as the ANOVA between them. The file is found in the currently working folder, and has the same name as the currently loaded data set, with the string '_anova' appended.

Save Means - first saves the mean of all groups into a new file appended with the string '_mean'. Then loads that file into memory.

Save Hist Data - saves the histogram values for the current parameter examined and for the currently selected group. The output file is name of the current data file appended with the name of the selected parameter, and the selected group, then '_histdata'

Save Percent - for each group, looks in the current parameter and counts the number of data points selected by the user, calculates what percentage of all values they constitute and saves the result in a new file named after the current working file, appended with the currently selected parameter and '_percent'.

10. 14. Keyboard shortcuts

1,2,3,4 - set view

space - clear the selected group
delete - remove the selected group

a - show anova
b - bold
m - toggle SEM/STD
p - toggle paired t-test
t - toggle show t-test
v - toggle vertical name plot
y - set Y axis

z - move group left
x - move group right

, - plot previous parameter
. - plot next parameter

11. Analyzing Data

11. 1. Analyzing Movement Frequency

11. 2. Analyzing Movement Directionality

11. 3. Analyzing Movement Kinematics

11. 3. 1. C-Bend and Counterbend Kinematics

11. 3. 2. Swim Kinematics

1. Select the type of maneuver whose kinematics you wish to analyze by checking Behavior → [Select scoot/burst/rturn etc]

2. Remove swim bouts with zero or one tailflip - there is no information to analyze in these swim episodes by checking Behavior→SwCycles

3. Select the kinematic parameter of interest.

Note, when analyzing the number of half swim cycles, it is important to remember that in instances where the fish was still swimming at the end of the recording, *Flote* designates the number of swim cycles as a negative number. There are two ways to deal with this:
1. Enter histogram mode, and select only those swim cycle values >1 then click the /Sel/ button to remove all the incomplete swim cycles.
2. Click the 'Abs' box on the left side of the menu area. This will convert all negative values into the positive value (eg, if the number of swim cycles is negative 5, it will become positive 5).
In situations where there is a very large proportion of incomplete swim bouts (such as where you use a small recording window, or where the larvae are extremely hyperactive), neither method is ideal for analyzing the number of swim cycles and you should be careful to present the data as the number of swim cycles observed NOT the actual number of swim cycles the larvae executed.

12. Other Applications

12. 1. Analyzing long time series data

For tracking, Flote loads a series of frames into memory, then performs tracking, identifying all the larvae in the initial frame, designating each with an identifying number then tracking each larva across all frames loaded into memory. It is also possibly to track very long time series, without uniquely identifying the path of all the larvae in the recording. For example, you may have a 15 minute video, recorded at a low frame rate (for example 50 frames per second, so a total of 45000 frames), with larvae continuously entering and exiting from the recording field. To produce a picture of the paths made by the larvae during this recording, setup batch analysis to track 45000 events of 1 frame. The tracking directory will then contain 45000 tracking files. You can use the Tracking→Convert tracks to text function to convert the tracks to a series of tab-separated text files containing the x,y positions of the larvae in each frame. Set the Tracking→Convert tracks to single text option so that all the tab-separated x,y positions are gathered into a single output file: C:\ftrack\alltracks.txt.

Open this file with a plotting program and generate a scatter graph of the x,y coordinates. So long as your frame rate is fast enough, this should produce an image containing all the tracks of all the larvae entering or leaving the recording field over the 15 minutes.

Note that if you have only a single larva in the testing arena, you can easily calculate information about the speed of movement using a spreadsheet.

13. Troubleshooting

I can't load files into Flote.

Solution 1. Try turning on the 'File→Alphanumeric file order' option. If files now load ok, then your naming convention is not exactly what Flote is looking for. You can still operate Flote normally, but it will take longer to load each set of frames.

Solution 2. There is a known bug where if you have certain windows open, the file load function fails. This often occurs if you have the zoom window or the time-series graph window open. So close these windows and try loading files again.

Tracking is very slow.
Make sure than 'Tracking→Rapid tracking' is turned on.

Too many heads are being found.

1. Make sure the fish are the only contrast elements in the field
2. Either reduce the size of the image so that the fish are of about the recommended size (ie about 50 pixels in length)
3. Play with the head centroid finding controls as described in section 3.1 to fit your image.