Chapter 2

Videograms: A Method for Repeatable Unbiased Quantitative Behavioral Analysis Without Scoring or Tracking

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Abstract

We present a method that complements both scoring by observers and automated tracking methods for quantifying behaviors. Based on standard motion enhancement, our algorithm converts a behavioral video recording into a single image ('videogram') that maps the spatial distribution of activity in the video sequence. This videogram can be used as a visual summary of activity and also as a direct, repeatable, and unbiased measure of animal activity. We describe the algorithm, and then use videograms to show acquisition of odorant-dependent place-conditioning in zebrafish trained in groups. We also demonstrate its potential for determining depth preferences and swimming speeds. This method generates activity measurements suitable for continuous variable statistics, and can be considered as an analysis alternative to behavioral tracking (over which it can have several advantages) for experiments not requiring exact trajectories.

Key words: Videogram, quantitative analysis, animal activity, place conditioning, depth preference, swimming speed.

1. Introduction

Quantitative analysis of animal behaviors is an important tool in zebrafish and other animal research (1, 2). Acquiring measurements from behavioral observation or video sequences has previously been based on manual scoring, e.g., (3–6) or tracking the behaviors, e.g., (7–12). Our goal here is to describe an alternative method for acquiring quantitative data that may be useful in behavioral experiments (with zebrafish or other animals).

A range of factors can be considered when choosing a behavioral analysis method. Scoring behaviors based on predetermined

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criteria creates quantitative data suitable for statistical analysis and thus objective assessment of behavioral responses to different treatments, e.g., (3–6). However, data from scoring are often categorical, and thus are unable to differentiate amongst subtle variations in behaviors, and also limit the range of applicable statistical tests. Moreover, scoring can be subject to observer bias and is often time-intensive. In particular, the time invested in scoring more than a few criteria is often prohibitive, and therefore restricts the range of metrics used to analyze behaviors. On the other hand, tracking behaving animals or their body parts creates excellent datasets that are both usable with continuous variable statistics and flexible with regard to analysis metrics, e.g., (9–13). However, tracking animals manually is especially laborious, and automatic tracking systems require stringent image quality regulation (since a unique object needs to be identified for tracking in each frame and mistakenly tracked objects can cause large deviations in tracks), are computationally intensive, and commercial packages are expensive. Moreover, many automated tracking systems cannot handle multiple animals if the possibility exists for their tracks to cross, although custom algorithms and software have been developed to overcome this problem, e.g., (14).

To complement these existing methods, we have developed an algorithm to reduce a video sequence into a single image (a "videogram") that measures the spatial arrangement of activity levels in the sequence. We employ standard motion enhancement, e.g., (7, 10, 15, 16) subtracting a background image from each video frame. The resulting images show lighter moving objects on a dark background. We then use a threshold to convert each to a binary image with white areas of activity in an otherwise black field (the subtracted background). However, rather than tracking the location of those white regions, we sum the images to create a spatial map of activity over the entire video sequence. The result is an image (the videogram) with lower intensity (darker) areas that had little or no activity during the video sequence, and higher intensity (lighter) areas that had more activity. This intermediate option for quantitative behavioral analysis provides repeatable, unbiased video analysis and yields continuous variable metrics without the complications of individual tracking. Furthermore, videograms can be used for analysis of either individuals or groups (that is activity of the group as a whole, not the activity of multiple individuals within a group). The method is computationally simple, can process far more frames than manual observations, and can be implemented in common image-processing packages (Matlab, ImageJ, Python, etc.). Here, we describe how to create a videogram from a behavioral video sequence, and offer optimization and troubleshooting tips. We then demonstrate its use by showing acquisition of odorant-dependent place-conditioning in groups of adult male zebrafish,

as well as brief examples of depth preference and swimming speed analyses.

2. Materials

2.1. Equipment

1. Digital video recording equipment.
2. A personal computer and image processing software.

2.2. Equipment Setup

The choice of camera and digital video recording equipment depends primarily on the experimental setup. Videograms can be created from any resolution video sequence recorded at any frame rate, with any (or no) video compression. The only requirement is a digital video file that captures the behavior of interest.

The algorithm described below can be implemented in any image-processing program that provides basic arithmetic image manipulation functions. In addition, software that allows the use of macros or programming will usually be highly advantageous (e.g., ImageJ, National Institutes of Health; Matlab, Mathworks, Inc.; Python, Python Software Foundation; etc.), although it could also be executed manually in programs such as Photoshop (Adobe Systems, Inc). In addition, a utility to convert a color video sequence to grayscale and to convert the video sequence to a series of images may be needed (e.g., VirtualDub, virtualdub.org; iMovie, Apple, Inc.).

3. Procedure

Videograms can be created from grayscale digital behavioral video sequences of one or more animals, measuring either individual or group activity, respectively (**Fig. 2.1**).

CAUTION: A high contrast source video sequence with no contaminating movements is important. Ideally, the animal(s) should be consistently darker or lighter than the background and they should be the only moving objects in the video sequence, although some deviations from this ideal are surmountable.

CAUTION: Videograms created from thousands of frames will likely need to be created using frame-by-frame processing rather than the simpler all-frames-at-once procedure presented here (see **Section 4.4** below).

The following steps create a videogram.

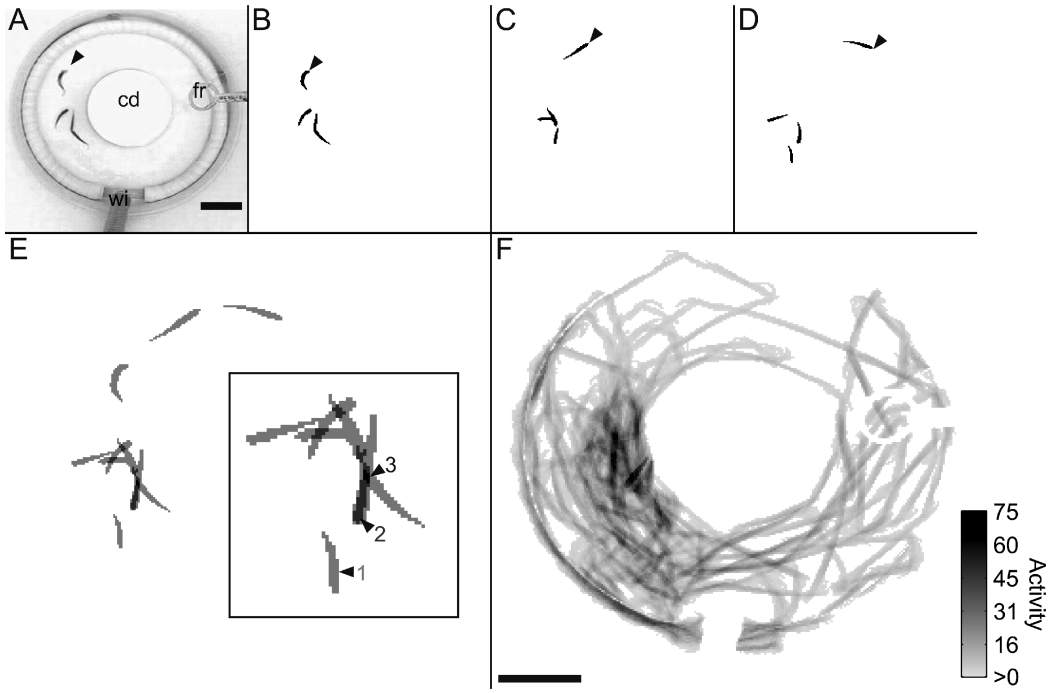


Fig. 2.1. Videogram creation. (**a**). A single frame from a video sequence of four zebrafish (one indicated, *arrowhead*) in an odor conditioning experiment (17). The camera is *above* a circular tank with a water inflow tube (wi), covered drain (cd), and a feeding ring (fr). (**b–d**). The same video frame and two subsequent frames (separated by 0.13 s) after background subtraction and application of a threshold, showing the inverted binary images of the four zebrafish (the same fish is indicated in each frame, *arrowhead*). *Black* pixels are indicative of activity at that location in that frame, since stationary fish would not be measured. (**e**). A videogram created by summing the frames in **b, c**, and **d**, with pixel intensities scaled to indicate activity levels. Inset shows how pixels occupied by a fish in just one frame (1) are 33% *gray*, in two frames (2) are 66% *gray*, and all three frames (3) are *black*. (**f**). A videogram of the entire video sequence, showing the distribution of activity in the tank. Activity scale: activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bars: 4 cm, shown in **a** for **a–d** and **f** for **e** and **f**.

NOTE: A demonstration of the procedure in ImageJ is available (*see* **Appendix**), as well as a more complex and versatile implementation in Matlab available upon request.

1. Convert the video sequence to a series of grayscale images, using a conversion utility if necessary. Uncompressed image formats are preferable since they do not blur contrast with compression. The video sequence is now a series of images, each with a rectangular array of pixels. Each pixel has an intensity representing its gray value, typically between 0 (black) and 255 (white), although greater bit-depth systems will also work. For example, an image of a zebrafish in a tank may have darker fish (pixel intensities ~50) swimming in front of a lighter background (pixel intensities ~200).

2. **CRITICAL STEP**: Ensure the moving animal of interest in the video sequence is lighter than the background. If the

animal is darker than the background (this is usually the case for zebrafish), invert all the images, reversing the grayscale.

3. Create a background image using one of three options:
   a. Option 1: use an image from a baseline portion of video sequence without any animals present (e.g., recorded before fish are introduced into the tank).
   b. Option 2: use an "absolute" mean image calculated from the entire behavioral video sequence. A subset of the frames can be used, provided the animal is not visible in the mean image.
   c. Option 3: use a "running" mean image calculated from a number of frames before and after the frame of interest.

   Any of these options can work successfully. Theoretically, a baseline image works best. However, practically an absolute mean image is the easiest to acquire, and a running mean image may be the only option if a dynamic background is present (*see* **Section 4**).
4. Create a series of subtraction images. Subtract the background image from each video frame image. Any regions of a video frame that are the same as the background will disappear (i.e., the pixel intensities are identical, and thus the subtracted image pixel intensities will be zero). Similarly, any regions darker (i.e., lower pixel intensities) will also disappear. Only regions of the video frame image that are lighter than the background image will have a pixel intensity greater than zero in the subtracted image. Thus, lighter moving objects (e.g., a swimming zebrafish in an inverted video sequence, *see* Step 2) will be the only objects visible in the subtracted images.
5. Create a series of binary images by applying a threshold to the subtraction images. Choose a threshold pixel intensity that separates the moving object of interest (e.g., the zebrafish) from any background noise. Importantly, the original video sequence must have enough contrast to consistently separate large fluctuations in pixel intensity caused by the animal, and small fluctuations in pixel intensity, created by the video camera and/or digitization process. The series of binary images now contain white regions with a pixel intensity of one, representing areas of activity (e.g., a swimming zebrafish) and black regions with a pixel intensity of zero, without activity.
6. Sum the series of binary images. The video sequence has now been converted to a single image, where the pixel intensity represents the number of frames during which activity occurred in that pixel. Black regions with zero pixel intensity show where no activity occurred in any of the binary

frames. Higher pixel intensity values show where more activity occurred (e.g., where the zebrafish swam more often).

Once a videogram is created, the pixel intensity represents the frequency of activity in that pixel's location over the entire video sequence. The videogram pixel intensity is equal to the number of frames for which the source video was higher than the threshold intensity, and thus provided only the moving animal is above threshold, the videogram pixel intensity measures how often the animal occupied that pixel location. If a baseline image without the animal present is used for subtraction (step 4), the pixel intensity measures occupancy. Alternatively, if a mean image is used for subtraction, the algorithm relies on motion (a motionless fish would produce a black videogram) and thus the pixel intensity measures activity (not occupancy). This occupancy or activity measurement is true whether a single animal or multiple animals were recorded in the original video sequence. In the latter case, the videogram simply represents the activity of the group of animals.

IMPORTANT: For display purposes, the pixel intensities will usually need to be normalized to a standard gray scale to avoid saturation. The videogram can then be used for a qualitative demonstration of the spatial distribution of activity (**Fig. 2.1**). Conversion into a quantitative behavioral measure will depend on the source video sequence and the activity being measured. For example, if the video sequence shows a zebrafish in a tank, the mean depth occupied by the fish can be calculated by using all pixel intensities as weights for a weighted mean of the vertical pixel coordinates (**Fig. 2.2**). Alternatively, if the zebrafish are subjected to treatments that may attract them to a location in a tank, then the mean pixel intensity in that region is a direct measure of the animal's presence in that region (**Fig. 2.1**). These are just two examples, but the range of possibilities for such measures is limited only by what can be captured in a video sequence and the algebraic manipulation of pixel intensities and coordinates.

4. Optimization

4.1. Source Video Sequence

The quality of the source video sequence affects whether a videogram accurately measures activity. The resolution and compression algorithm used in the source video sequence are important only insofar as they affect whether or not the behavior is still visible in the video sequence. However, contrast between the animal and the background is paramount, since areas where the animal has similar gray values to the background cannot be analyzed.

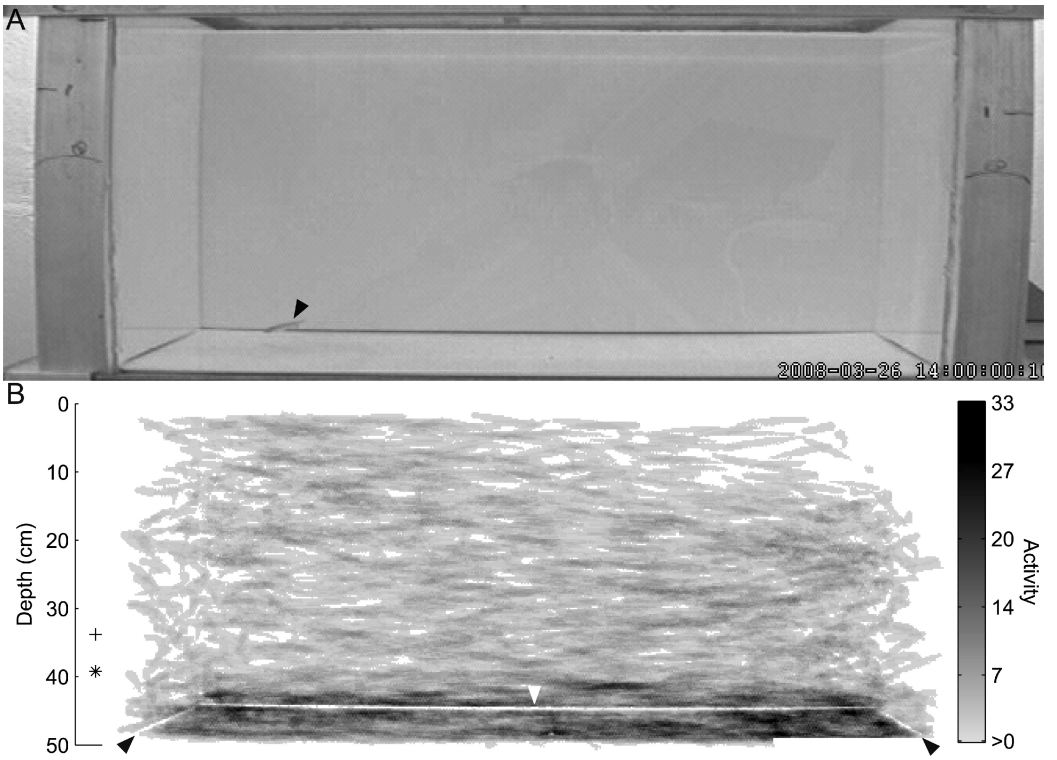


Fig. 2.2. A videogram used to measure depth preference of zebrafish. (**a**). A single frame from a video sequence of one zebrafish (*arrowhead*). (**b**). Videogram showing how the distribution of activity is concentrated toward the *bottom* of the tank, as expected for a zebrafish newly introduced to a tank. The distinct lines of missing activity (*arrowheads*) are due to the close match between the pixel intensities of the zebrafish and tank joint. These lines also emphasize the effect of parallax on a two-dimensional videogram, which conflates different depths in the three dimensional tank. Analysis of the video time stamp (*lower right*, **a**) was excluded using a region of interest. Activity scale: activity frequency over 1 h, sampled at 1 frame s^{-1}. Inset: mean (+) and median (∗) depth of the zebrafish calculated from the activity values (i.e., frequencies) and vertical pixel coordinates of the videogram.

Depending on the experiment, small regions of low contrast may be inconsequential (**Fig. 2.2**), as they may only be a small proportion of the recorded activity (e.g., if a zebrafish swims in front of tank joint and "disappears" in a still video frame). However, careful choice of both lighting and background materials (e.g., lining three sides and the bottom of a zebrafish tank with white Plexiglas) will greatly improve consistent detection of activity. If color provides the best contrast, then the algorithm can be modified to use red, green, blue, hue, or saturation values in place of grayscale intensities in Step 1. In addition, the animals must be the only moving objects in the video sequence. Any movements created by the experimenter, abrupt changes in background, reflections (e.g., zebrafish reflected from underside of the water surface) cannot be distinguished from animal activity. Cameras and lights should therefore be placed to avoid contamination by extraneous movements.

Videograms, similar to tracking, are subject to the disadvantages of using a two-dimensional view of a three-dimensional behavior. For example, when tracking zebrafish depth preferences, parallax causes different tank depths to appear at the same position in both single video frames and videograms (**Fig. 2.2**). These problems are common to all video analysis methods, and can be eliminated by employing multiple cameras or mirrors or mitigated by choosing camera positions and lenses that minimize parallax.

4.2. Video Frame Rates and Durations

Choosing frame rates and durations depend on the behavior under analysis. Faster frame rates create track shapes that show entire movements. For example, in zebrafish, a faster swimming animal will create a longer, but less intense track of non-zero pixels in a videogram than a slower moving animal (**Fig. 2.3**). The intensity and track area values can then be used as measures of swimming speed without ever tracking the fish. Alternatively, if the videogram itself is then converted into a binary image, standard analysis methods can be used to calculate the dimensions of such a region, and thus swim speed (or other locomotory

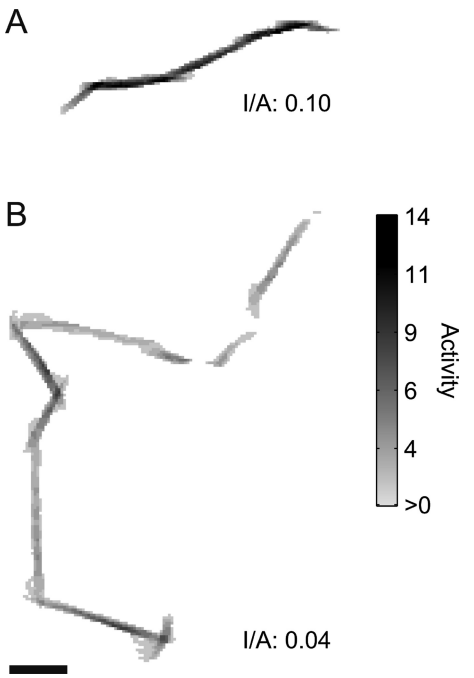


Fig. 2.3. Two videograms distinguish slow and fast swimming zebrafish. **a**. A slowly swimming fish creates a relatively short movement trace with high pixel intensities. **b**. A fast swimming fish creates a relatively long movement trace with gaps and low pixel intensities. An intensity to area ratio quantifies the difference between slow and fast swimming fish (I/A = summed intensities of all non-zero pixels/number of non-zero pixels, activity pixel^{-1}). Activity scale: activity frequency over 2 s, sampled at 30 frame s^{-1}. Scale bar: 2 cm.

variables such as the acuteness of turns) can be directly calculated, still without any tracking. Slower frame rates sample the activity at intervals. If these are used over longer duration video sequences, then the general location of activity will be depicted by the videogram (**Figs. 2.1** and **2.2**).

4.3. Region of Interest (ROI)

An ROI can be used to exclude certain areas of the video sequence, or alternatively, include only certain areas. For example, video time stamps and extraneous motion around the periphery can be excluded using an ROI (**Fig. 2.2**). Alternatively, ROIs can restrict analysis to the areas where the behaviors of interest occur or create multiple individual videograms for multiple animals in a single frame.

4.4. Processing Speed

Three primary techniques can reduce processing times. An ROI can be used to crop the pixel dimensions of every image in the series, reducing the total number of pixels processed. Frame-by-frame processing can also be beneficial or essential for large numbers of frames that cannot be simultaneously loaded into computer memory. Rather than applying each step of the algorithm to all frames before moving on to the next step, a running summed binary image is kept as the steps are applied to each frame in sequence. This enhances speed because it avoids loading all images into computer memory simultaneously, and also allows examination of the effect of different settings without processing all frames. Furthermore, since single frames can usually be processed entirely in computer memory, it can also be used to reduce the number of files written to hard disk, often a strong contributor to processing time (although this eliminates the possibility of reviewing the various steps of the algorithm and will thus reduce troubleshooting options). Finally, longer durations and higher frame rates increase processing time, and if these can be reduced without compromising the capture of the behavior, then shorter calculations are possible. For example, depth preference measurements at 30 frames s^{-1} generate 108,000 samples h^{-1}, yet provide similar depth information (data not shown) as a videogram based on 3,600 samples generated from at 1 frame s^{-1} (**Fig. 2.2**).

4.5. Comparing and Combining Videograms

Comparing and combining videograms add an additional requirement that the individual videograms be scaled similarly. This accounts for both variations in video sequence duration or frame rate and also the possibility of dropped frames during the digitization process. Dividing pixel intensities by the number of frames in the source video sequence standardizes the videograms to activity $frame^{-1}$, and allowing comparison amongst all video sequences recorded using the same video setup. Standardized videograms can also be averaged to examine activity pooled from multiple video sequences (**Fig. 2.4**). In this case, provided the video

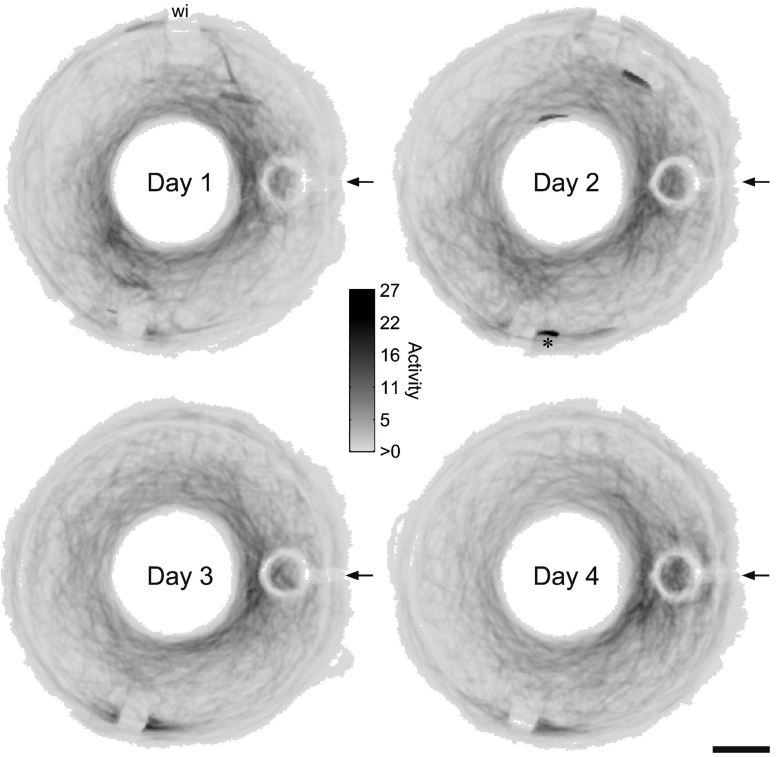


Fig. 2.4. Averaged videograms show the acquisition of an odorant-dependent place preference by zebrafish trained in groups. Six groups of 4 fish were trained over 4 days to associate an odor with food provided inside a feeding ring (*arrows*) see (17) for details. Videograms were created from video sequences showing the fish behaviors after odor presentation (conditioned stimulus), but before food reward administration (unconditioned stimulus). Videograms from 3 trials per day for each group of fish were mapped onto a common coordinate system with the same feeding ring location, and then averaged across all six groups. The concentration of activity near the feeding ring after odor presentation increased each day (as opposed to the opposite side of the tank, which showed decreasing activity). Distinct areas of reduced activity are due to water inflow tubes with varying locations across the six training groups (one indicated, wi). Distinct areas of high activity, particularly on day 2 (∗) are a result of fish 'hiding' in certain locations of the tank due to dominance behaviors in one group. Activity scale: average activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bar: 4 cm.

sequences can be registered by mapping to a common coordinate system, the video sequences need not be taken with same camera nor even be of the same scene.

5. Trouble shooting

The easiest method to confirm a videogram accurately depicts animal activity alone is to create a video sequence from the series of binary images (Step 5). Watching the source video sequence

followed by the binary video sequence will highlight any anomalous areas that are measured as activity but are not created by the animal(s). In the case of zebrafish, this can often be reflections, air bubbles, the water meniscus, camera or tank movements caused by clumsy experimenters, or simply random noise created by pixel fluctuations. If these cannot be eliminated by optimizing the source video sequence, several processing methods can be used to filter them from the videogram. Digital image filters (e.g., a median filter) can be used to remove random white noise from subtraction images (Step 4). Adaptive thresholds, adjusted based on the total pixel intensity of an image can allow creation of consistent binary images (Step 5) despite fluctuations in lighting (for example, if lighting alternates between visible and infrared illumination). Alternatively, standard image processing methods allow statistics (dimensions, area, concavity, etc.) to be gathered on all objects in a binary image (an object is a contiguous area of white pixels). If spurious activity regions have consistently different shapes from those generated by the moving animals, then these object statistics can be used to select and erase them from the binary image series (16).

Dynamic backgrounds are another factor that can compromise the accuracy of a videogram. However, careful choice of video frame rate and the frames used to calculate a mean image as a background image (Step 3) used in image subtraction (Step 4) can circumvent this problem. As long as the background changes more slowly than the animals move, then a mean image that is calculated relative to the frame being processed should be able to highlight animal activity alone. The key is to select frames at intervals both before and after the frame being processed such that the animal's activity is blurred into the background, while that averaged background still resembles the background in the frame being processed (for example, every 5th frame from the 25th frame preceding frame to the 25th frame following). Alternatively, if there are slight changes in background and foreground between video sequences (particularly as consequence of a different camera position), image registration can be used to transform the videograms to a common map, allowing comparisons to be made accurately.

Finally, if the contrast of the moving animal is dynamic, with either higher or lower pixel intensity than the background, then clipping of activity can occur. For example, if calculations are designed to detect a dark zebrafish moving over a light background, then no activity will be detected if the fish moves to an area where it appears lighter than the background. In this case, an absolute value subtraction image can be created (or the sum of two subtraction images: the frame minus the background and the inverted frame minus the background). This will enhance activity with either higher or lower pixel intensity in the source video

sequence, but will be unable to enhance activity in regions where the contrast is in transition. Moreover, the subtraction images will be inherently noisier, and post-processing increase of the likelihood will be needed.

6. Anticipated Results

Videograms can be used for both qualitative observations of large video data sets as well as quantitative analysis. For example, we implemented our videogram algorithm in Matlab (source code available upon request) to examine the acquisition of odorant-dependent place-conditioning during group training of zebrafish (**Fig. 2.1**). Braubach et al. (17) trained groups of fish to associate an odor (conditioned stimulus) with a food reward provided inside a feeding ring on one side of a circular tank (unconditioned stimulus). After training, individual animals spent more time near the feeding ring when odor stimuli were applied, and thus had developed an odorant-dependent place preference. We therefore reasoned that the training video sequences should show the progressive acquisition of this place preference, without the need for tracking individual fish within the groups. To examine the change in fish behavior, we created an averaged videogram for each day of training (**Fig. 2.4**). Combining data from three 30 s training trials per day for six groups of four fish, each averaged videogram provides an unbiased objective analysis of 16,200 video frames. They demonstrate how on the first day the fish do not concentrate their activity near the feeding ring when exposed to the conditioned odorant. However, on each subsequent day the fish activity distribution is increasingly biased toward the feeding ring. Although this trend is not as consistent when measuring the total activity within 6 cm of the ring (**Fig. 2.5**), if activity is measured as a proportion of the total over the entire tank (a better measure of the any place preference, in our view), a linear regression over the conditioning period showed a significantly ($R^2 = 0.18$, $F_{1,22} = 5.1$, $P = 0.035$) increasing proportion of activity that occurred within a 6 cm radius of the feeding ring center (**Fig. 2.6**). Thus, we are able to use videograms to show the changes in behaviors captured in video sequences from multiple cameras on multiple days, and also to find quantitative evidence that that odorant-dependent conditioning can occur in groups of zebrafish trained together.

Videograms are versatile and can be used with almost any behavioral video sequence with reasonably consistent contrast. Both the location and level of activity in the videogram can be measured, allowing the calculation of spatial preferences and other behavioral parameters. For example, a slow swimming

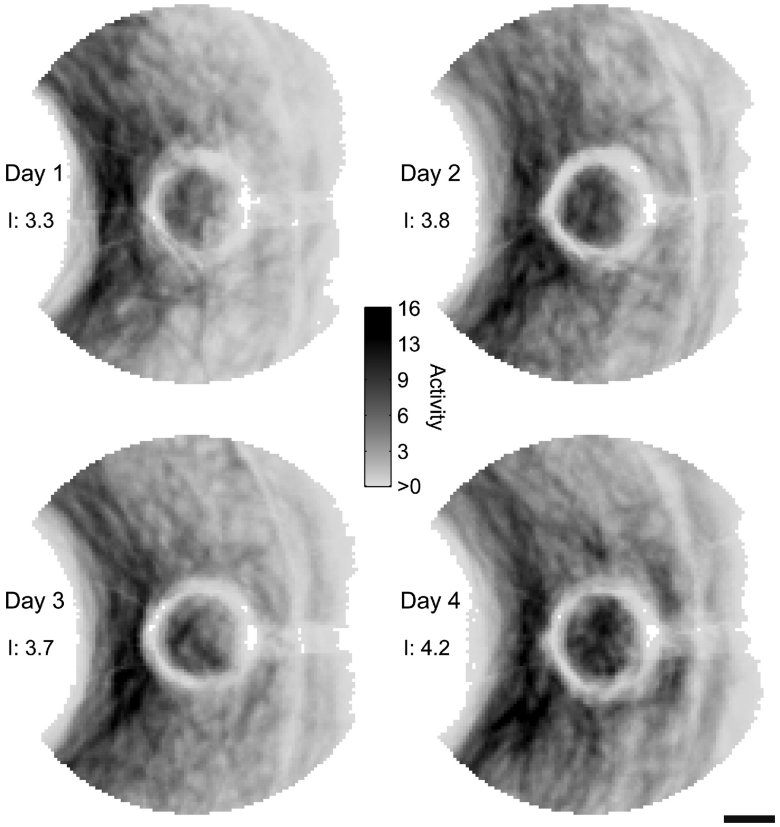


Fig. 2.5. Magnified averaged videograms (*see* **Fig. 2.4**) showing only the analysis regions around the feeding rings. Averaged across the six groups of 4 zebrafish, the total activity within 6 cm of the centre of the ring after odor presentation increased between days 1 and 2, showed a small decrease between days 2 and 3, and increased again between days 3 and 4 (I, summed mean activity pixel^{-1}). Activity scale: average activity frequency over 30 s, sampled at 30 frames s^{-1}. Scale bar: 2 cm.

zebrafish creates a short, bright videogram whereas a fast swimming zebrafish creates a long dim videogram (the binary images of the slow swimming fish overlap more between frames, and thus the videogram trace has a high intensity but a smaller area after summation, whereas the fast swimming fish has less overlap and thus lower intensity and larger area). Accordingly, an intensity: area ratio provides a convenient metric for distinguishing slow versus fast swimming fish (**Fig. 2.3**). Based on both location and intensity measures, we have used videograms to measure depth preferences during tank acclimation (**Fig. 2.2**), analysis of swimming speeds or trajectories (**Fig. 2.3**), startle responses (Stoyek and Croll, in prep.), or analysis of larval olfactory behaviors (Braubach, Fine and Croll, in prep.). Yet other behavioral parameters can be measured based on further analysis of the videogram. Since the videogram is an image showing activity levels, a threshold can be applied to convert it into a binary image, with a black background of low activity (below the threshold) and a white

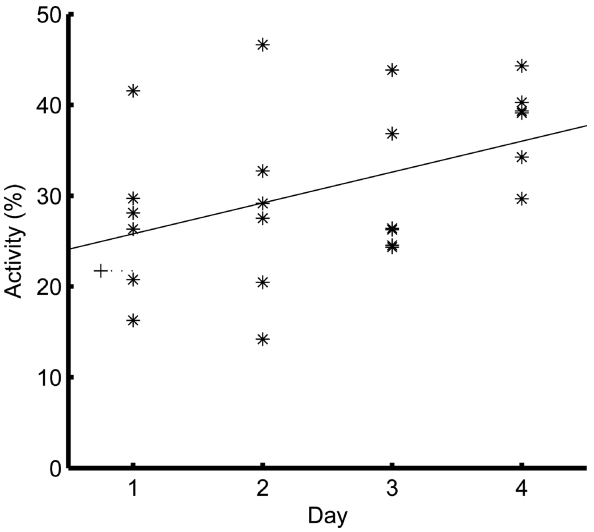


Fig. 2.6. Activity calculated from videograms shows the acquisition of odorant-dependent place-conditioning by zebrafish trained in groups. Six groups of 4 fish were trained separately over 4 days to associate an odor with food provided inside a feeding ring, see (17) for details. The sum of activity within 6 cm of the ring centre as a proportion of all activity in the tank was averaged from videograms of 3 trials per day for each group of fish. Initially, activity near the ring was similar from what would be expected by chance (+ indicates baseline activity before training began, averaged from single videograms of each group of fish). However, a linear regression over time (*solid line*, $P = 0.035$) shows a significant increase in activity close to the ring indicative of place-conditioning.

region of high activity (above the threshold). Commonly available image analysis methods can quantify the shape of the high activity region (see the documentation for ImageJ, Matlab Image Processing Toolbox, etc.), enabling measurements of speed (e.g., the white region feret/duration of video sequence), turn angle (difference between the angles of the major axes of two ellipses fit to two white regions from sequential videograms created just before and after a turn), tortuosity (aspect ratio of an ellipse fit to the region), etc. Furthermore, videograms can be used for analysis of other types of experiments as well. Physiological analysis of breathing movements, eye movements, or any other movement that can be captured on video sequence with consistent contrast can be measured with a videogram. Finally, if color figures are an option, we have found an overlay of a pseudocolored videogram on top of the background image from the video sequence to be a striking in-context demonstration of the activity distribution (8).

The activity patterns shown by videograms are similar but not identical to position traces created by tracking algorithms. A videogram shows the average activity distribution of the animal(s) over the video sequence, whereas a behavioral track is a continuous series of individual locations. Thus, for long tracks where the animal(s) repeatedly occupy the same location and the

track points become crowded and overlap, a videogram is better at showing the relative distribution of activity. (Note that tracking data could be converted to an image very similar to a videogram by mapping location frequencies to pixel intensity; however, this makes tracking redundant.) Moreover, errors in isolating the moving animal during the image processing steps have less of an effect on videograms (depicting averaged data) than tracks (depicting unitary data points).

This difference in error susceptibility and several other factors make the image quality requirements for videograms less stringent than for tracking in video sequences. Both videograms and tracking use background subtraction followed by application of a threshold to create a binary image, and thus both methods require consistent contrast and brightness. However, tracking algorithms must identify a single white region in the binary image created from each frame, requiring absolutely consistent pixel intensity contrast or alternatively an algorithm that handles two possibilities: (1) the animal "disappears" below threshold, and thus no white region is present and the frame must be skipped; or (2) the animal is represented by multiple white regions created by contrast fluctuations across the animal, and thus a filter must select one region or combine the multiple regions for successful tracking. Furthermore, extraneous white regions in the binary image (those not representing the animal) must be avoided entirely or filtered from each frame (by position, size, shape, etc.) for tracking to succeed. In contrast, the algorithm for videogram calculation requires no modification to handle frames where the animal "disappears;" and provided these are infrequent, the videogram will still accurately represent the spatial distribution of activity (the benefit of showing averaged data). Videogram calculation is also unaffected by multiple white regions due to pixel intensity fluctuations, and can still accurately represent animal's activity in a video sequence without filtering such fluctuations. Similarly, extraneous white regions can be rendered negligible by averaging sufficient frame numbers without extraneous regions, or they can be filtered from the final videogram (not necessarily every frame). Thus, the image quality requirements for consistent brightness and contrast, although still present, are considerably lower for creating videograms than tracking animals. Moreover, the complexity of the algorithm (and the programming code for automated analysis) is lower for videograms than tracking. Commercial software packages with tracking algorithms typically have a number of algorithms to handle contrast inconsistencies, but we suggest researchers requiring more economical options, custom analyses, or integration with other experimental requirements, and thus coding their own software, should consider the use of videograms.

In summary, we suggest videograms are a useful option for behavioral video analysis to be considered along with scoring and

tracking. Once the algorithm is optimized for a particular experiment and confirmed through pilot video sequences to accurately capture the activity of interest, videogram creation can be completely automated in an unbiased and repeatable fashion. This can allow both more extensive and more accurate analysis than scoring by observers. Videogram measurements are thus comparable to tracking data. Yet tracking requires more stringent contrast control since mistakes in tracking can result in large path deviations, whereas similar rare events have little effect on videograms calculated from many frames. Moreover, since most tracking algorithms rely on binary images to identify the location of animals being tracked, both videograms and tracking can be accomplished with considerable overlap in image processing. Thus, videograms can be used for both qualitative observation and quantitative measurement of behavioral video sequences, and complement either scoring or tracking of behaviors in experiments.

7. Appendix: Using ImageJ to Create a Videogram

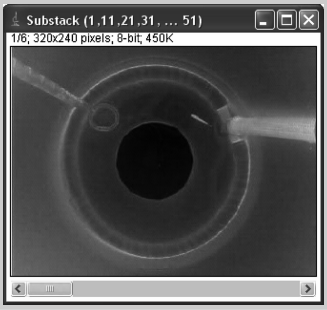
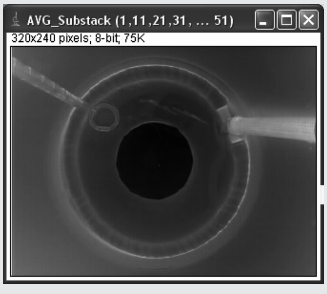
This document outlines a step-by-step procedure to produce a videogram from a short sample movie.

7.1. Requirements/ Preparation

1. **ImageJ**
   The procedure uses the MacBiophotonics ImageJ release, which bundles a number of necessary plugins (AVI Reader, Substack Maker, Handle Extra File Types) http://www.macbiophotonics.ca/imagej/
2. **Sample video**
   The procedure relies on the movie being opened directly in ImageJ. This only works if the movie is uncompressed. Therefore, do any *one* of the following:
   - Use an uncompressed AVI movie and load it into ImageJ using the AVI Reader plugin http://rsbweb.nih.gov/ij/plugins/avi-reader.html
   - Use a compressed movie and convert it to an uncompressed movie using another video processing program, and use the AVI Reader plugin.
   - Use a compressed movie and convert it to a series of uncompressed (TIFF, TARGA, BMP, etc.) images using another video-processing program, and then use the File: Import: Image Sequence. . . command in ImageJ to create a stack of grayscale images from the series of image files.

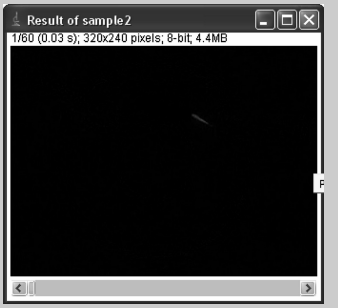
   The sample movie used in this example is available: http://people.stfx.ca/rwyeth/vidsimages.html or contact Russell Wyeth rwyeth@stfx.ca

ImageJ commands to convert the uncompressed video "sample2.avi" into a videogram

Menu command in ImageJ *(v1.42 l, MacBiophotonics release)*	**Image Window Result**
1. File: Open browse and select "**sample2.avi**" **Open** *Only uncompressed AVI files can be opened by ImageJ.* First Frame: **1** Last Frame: **60** ☐ Use Virtual Stack ☑ Convert to Grayscale ☐ Flip Vertical **OK**	
2. Edit: Invert Process all 60 images? There is no undo if you select "Yes" **Yes**	
3. Plugins: Stacks – Reducing: Substack Maker Enter either range (e.g. 2–14) or a list (e.g., 7,9,25,27): **1,11,21,31,41,51** **OK** *This stack will be used to create the mean image.*	
4. Image: Stacks: Z Project. . . Start slice: **1**; Stop slice: **6** Projection Type: **Average Intensity** **OK** *This creates a poor mean image, with a considerable 'shadow' of the fish's motion, yet still suffices to demonstrate the method. A longer video providing more widely spaced frames (selected in step 3) would produce a mean image with little trace of the fish.*	

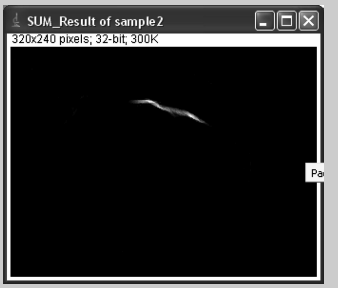
(continued)

5. Process: Image Calculator...
   Image 1: **Sample2.avi**
   Operation: **Subtract**
   Image 2: **AVG_Substack(1,11,21,31...51)**
   ☑ Create New Window
   ☐ 32 bit (float) Result
   OK
   Process all 60 images? ? There is no undo if you select "Yes"
   Yes

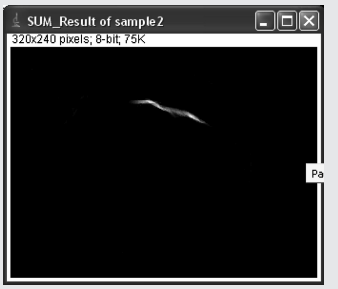


6. Image: Adjust: Threshold...
   [threshold minimum slider, top]: **20**
   [threshold maximum slider, middle]: **255**
   [threshold display, bottom]: **Red**
   Apply
   Convert all images in stack to binary?
   ☐ Calculate Threshold for Each Image
   ☑ Black Background
   OK

7. Image: Stacks: Z Project...
   Start slice: **1**;
   Stop slice: **60**
   Projection Type: **Sum Slices**
   OK



8. Image: Type:8 bit



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