Motility Analysis Macro v2.2

David P. Astling
Zusman Lab
UC Berkeley

June 2005

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About the Macro

This macro is a Visual Basic program for Microsoft Excel to analyze the motility of gliding bacterial cells. The macro uses the x,y coordinates of each cell from time lapse movies to calculate reversal frequency, average velocity, distance traveled, pauses and standard deviations in these measurements. x, y coordinates of each cell can be obtained with the motion tracking software Metamorph (Universal Imaging Corp., Downington, PA). For users who have obtained the x,y coordinates by some other means, a subprogram "DataFromXY" is included with the macro which will convert the x,y coordinates into the 'Metamorph' format.

- This Macro is freeware, however please site:

  Astling, DP, Lee, JY, Zusman, DR. (2005) Differential effects of chemoreceptor methylation-domain mutations on swarming and development in the social bacterium *Myxococcus xanthus*. Mol. Micro.

- If you make improvements or add additional features to this macro please share them with others.

- Please send questions, comments or bugs to: dastling@berkeley.edu

The Dataset Required

RevFreqMacro.xls

	A	B	C	D	E	F	G	H	I	J
1	Image Name	Object #	Frame #	X	Y	Distance	Velocity	Absolute Angle		
2	DZ2_6-24-03	1	1	0	0	0	0	0		
3	DZ2_6-24-03	1	2	0.1862	-2.208	2.2158	0.1477	-85.179		
4	DZ2_6-24-03	1	3	0.4757	-3.4725	1.2972	0.0865	-77.106		
5	DZ2_6-24-03	1	4	0.5998	-4.2828	0.8198	0.0547	-81.291		
6	DZ2_6-24-03	1	5	0.8317	-5.2928	1.0362	0.0691	-77.072		
7	DZ2_6-24-03	1	6	1.2282	-6.7364	1.4971	0.0998	-74.643		
8	DZ2_6-24-03	1	7	1.4895	-7.8082	1.1032	0.0735	-76.297		
9	DZ2_6-24-03	1	8	1.6003	-9.107	1.3036	0.0869	-85.124		
10	DZ2_6-24-03	1	9	1.6915	-9.8478	0.7464	0.0498	-82.979		

Summary | Results | Sheet1 | raw

Ready | Sum=0 | SCRL

Figure 1: A screenshot of a typical dataset

Column A: Name of the movie. This name must be in every row of this column.

Column B: Object Number. This refers to the cell tracked by Metamorph.

Column C: Frame Number. Refers to each frame of the movie analyzed.

Columns D and E: The position of the cell on the X-axis and Y-axis respectively. The orientation of the axis does not matter for the macro, however note that for Metamorph, a cell traveling down the screen is moving in the positive direction on the Y-axis. A cell moving to the right is moving in the positive direction in the x-axis. Also note that the units of this column are in pixels. The units can be converted to microns using a picture of a ruler or hemacytometer taken with your microscope setup.

Column F: The distance the cell moves per frame of the movie. The distance is the hypotenuse of a right triangle with Δx and Δy as the length of the two sides. The total distance that the cell moved during the course of the movie is the sum of all the values in this column.

Column G: The velocity of the cell (distance the cell moved per frame/time between frames). Note: sometimes Metamorph does not accurately calculate this value. The Macro recalculates this value by dividing the distance by the time per frame supplied by the user.

Column H: The angle of the direction of movement. The values range from -180 to +180 degrees. The orientation of the axis does not matter for this macro, however note that for Metamorph a cell moving down the screen will have an angle of +90 degrees. A cell moving toward the upper right corner of the screen will have an angle around -45 degrees.

Column I: The distance of the cell from the place where the cell started. This column is not used in the macro, but may be helpful to the user for some other purpose.

Note: Columns F through I can be generated by running the “DataFromXY” macro.

How to Use the Macro

1. Open the Excel file called 'MotilityMacro' and click "enable macros" if prompted.

2. Copy and paste the Metamorph data from your movie into the worksheet 'raw'.

3. Go to the upper right portion of worksheet 'Sheet1' and provide the appropriate information in column P. The velocity and angle thresholds are the minimum values required for reversals to be considered. If the cell moves slower than the velocity threshold, the cell is considered paused. Likewise if the angle made by the cell is greater than the threshold, it is considered as a reversal. **For your first time try an angle threshold of 110 and a velocity threshold of 1.5 pix/min.** The angle should be a number between 0 and 180 (see Figure 2 below). The values for the velocity threshold will depend on your microscope setup. With our setup 1.5 pix/min corresponds to 0.83 μm/min (using a 40X objective with 15 sec per frame).

4. To run the macro press Option+Apple+R (for PCs: Command+ALT+R).

   The data in the worksheet "raw" is copied into the worksheet "Sheet1" where the calculations are performed. Using the velocity threshold that you provided from step 3 above, the rows where a pauses occur are deleted. After deleting the pauses the calculations are performed. The calculated reversal frequencies and other parameters should appear in the spreadsheet called 'results'. See Figure 3:

5. To analyze the next set of data, simply clear the data in the worksheet "raw" and paste in the new information. There is no need to repeat step 3 above as long as the information is the same for both data sets. Alternatively: the last set of data can be reanalyzed by making the appropriate changes and rerunning the macro.

6. Run the macro again by pressing Option+Apple+R (or Command+ALT+R for PCs).

7. The results from the previous data set in the worksheet "Results" will be shifted down the appropriate number of rows and the new set of results will appear on top. This way multiple data sets can be analyzed sequentially without having clear the results each time. The oldest data will appear at the bottom.

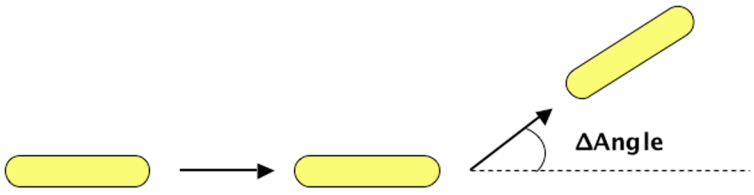
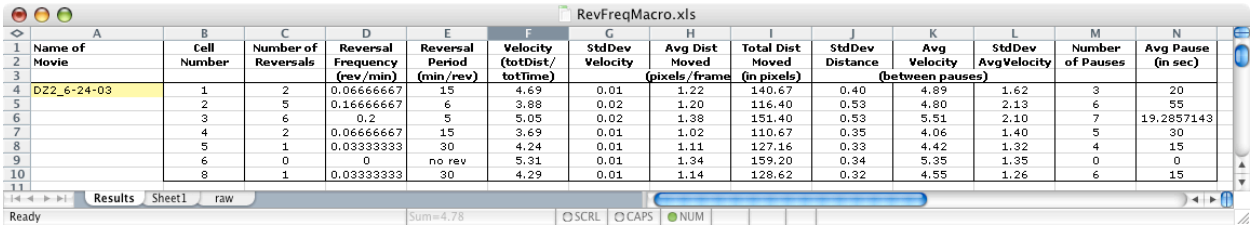


Figure 2: The change in angle

An Explanation of the Results



RevFreqMacro.xls

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Name of Movie	Cell Number	Number of Reversals	Reversal Frequency (rev/min)	Reversal Period (min/rev)	Velocity (totDist/ totTime)	StdDev Velocity	Avg Dist Moved (pixels/frame	Total Dist Moved (in pixels)	StdDev Distance	Avg Velocity (between pauses)	StdDev AvgVelocity	Number of Pauses	Avg Pause (in sec)
4	DZ2_6-24-03	1	2	0.06666667	15	4.69	0.01	1.22	140.67	0.40	4.89	1.62	3	20
5		2	5	0.16666667	6	3.88	0.02	1.20	116.40	0.53	4.80	2.13	6	55
6		3	6	0.2	5	5.05	0.02	1.38	151.40	0.53	5.51	2.10	7	19.2857143
7		4	2	0.06666667	15	3.69	0.01	1.02	110.67	0.35	4.06	1.40	5	30
8		5	1	0.03333333	30	4.24	0.01	1.11	127.16	0.33	4.42	1.32	4	15
9		6	0	0	no rev	5.31	0.01	1.34	159.20	0.34	5.35	1.35	0	0
10		8	1	0.03333333	30	4.29	0.01	1.14	128.62	0.32	4.55	1.26	6	15
11														

Results Sheet1 raw

Ready Sum=4.78 SCRL CAPS NUM

Figure 3: A screenshot of the Results page

The name of each movie is displayed in the first column and highlighted in yellow. This name is copied from the first column of the raw data. The cell number is displayed in the second column. The data for each cell is listed in each row. The calculations are a follows:

Number of reversals: 'no rev' means no reversals have occurred.

Reversal Frequency: [number of reversals]/[total time (in min)].

Reversal Period: [total time]/[number of reversals].

Velocity: Average velocity [total distance moved by the cell (in pixels)] / [total time]

StdDev Velocity: [standard Deviation in distance moved] / [total time]

Avg Dist Moved: The average distance moved. Average of column F in "Sheet 1."

Total Dist Moved: The sum of column F in "Sheet 1."

Avg Velocity: The average velocity between pauses. The average of column G in "Sheet 1". Note that this velocity will be higher than the other velocity measurement above. The user can decide which value to report.

Number of Pauses: The number of occurrences where the velocity was lower than the threshold.

Avg Pause: The average time for each pause (in sec). The average of Column L in "Sheet 1."

Evaluating the Quality of the Results:

The data listed in the worksheet "Results" is also listed to the right side of each cell in the worksheet "Sheet1" (see Figure 4). Scroll down to see the results for each cell. The reversal events are indicated with a "1" in column K. You can replay the movie and fast-forward to the indicated frame to verify the reversal event. The pauses are indicated with a number in column L. The number refers to the length of the pause (in sec).

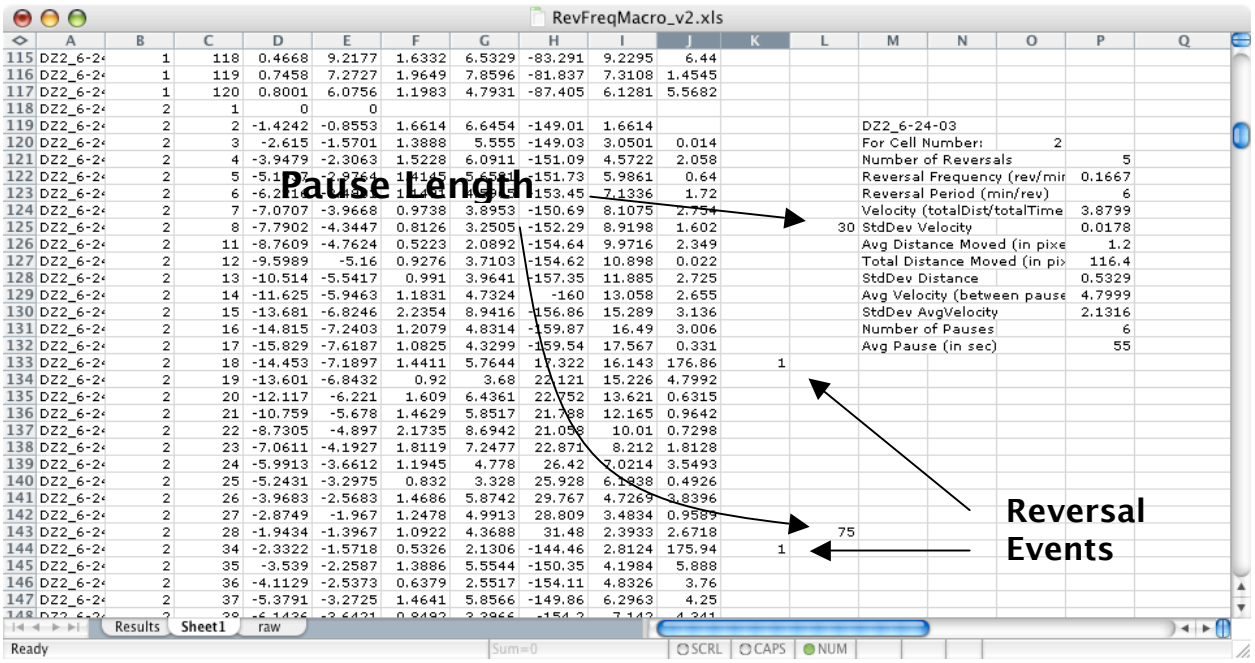


Figure 4: A screenshot of Sheet1 showing the calculated results, reversal events and pauses.

For your first time analyzing a set of data, a new strain or new culture conditions:

1. Return to the worksheet called "Sheet 1."
2. Compare the reversal frequencies in column K to actual reversals in your movie.
3. Adjust the angle and velocity thresholds for more accurate results

    - for fast moving cells, increase the velocity threshold (>4 pix/min)
    - for hyper reversing cells, lower the velocity threshold
    - for cells that wander, decrease the angle threshold
    - for cells that move in a straight line, increase the angle threshold

9. Change the values in Column P in worksheet "Sheet 1" and press Option+Apple+R to reanalyze the data.

10. If the cell has significantly long pauses, the data for that cell may have to be discarded. Look at the total distance moved and the pause lengths and reexamine the movie manually for those cells with significant pauses. In some cases the cell is simply moving slowly but has valid reversal behavior. In other cases the cell stops moving (or dies) halfway through the movie.

A Few Helpful Tips:

• IMPORTANT: A human will always be the best judge of reversal events. If you have trouble determining if a reversal event has occurred by manual playback, you cannot expect any macro to detect it.

• Cells must not be in contact with each other. Cell contact not only makes cell tracking difficult but also influences cellular behavior. Start the movie as early as possible and film cells that are two cell lengths apart.

• Cells that wander off the screen should be discounted

• Frame rate must be 3 to 4 times faster than the reversal period. For example, to film the *frzD* strain which reverses every 2 minutes, you must capture a frame every 15 sec in order to accurately observe reversals. The macro compares the angle two frames before and two frames after each reversal event. If reversals occur too frequently, then the macro will not detect them. Too many frames is much more desirable than not enough frames. There are two macros "averageXY" and "TooManyFrames" included which will average or subtract frames if too many have been taken.

A Few Helpful Utilities

• **lazyMacro:** This macro will save you a few keystrokes and is useful when analyzing a large number of movies. Open your dataset and press "Option+Apple+G" This subroutine will clear the old dataset in the "raw" spreadsheet, paste in your new dataset and run the macro for you. Be sure that window for your new data file is active and that the MotilityMacro is open.

• **DatafromXY:** This macro is needed if you lack the data in columns F through I. The macro uses the x,y coordinates to calculate the distance traveled per frame, the velocity and angle that the cell moved. To run the macro, press "Option+Apple+Y"

• **tooManyFrames:** Use this macro when your frame rate is too fast. The macro will delete every other line and recalculate the distances and angles.

• **averageXY:** This macro is similar to tooManyFrames except that it averages every other frame in the "raw" spreadsheet before deleting them. If you use this macro, follow up by running "DatafromXY."

• **subtractinitialXY:** This macro will set the initial coordinates of the cell to zero and adjust the other coordinates accordingly. This macro is handy when the x, y coordinates were obtained from a source other than Metamorph.

• **resetMacro:** This macro clears the data in all spreadsheets and restores the values in the upper right of Sheet 1 to the default values.

Extra Analysis Tools

1. Measure the actual time between reversals

Reversal frequency is often calculated by counting the number of reversal events over a period of time and dividing by the total time. However this may mask other useful information. For example a mutant strain may reverse frequently under certain circumstances but less frequently in other cases, resulting in a bimodal distribution of reversal frequencies. Or cells in population may reverse at different rates depending upon the context. A histogram of the time between reversals would reveal these patterns. The macro "TimePerRev" takes the data in Sheet 1 and calculates the time between reversals and reports the data in Sheet "TimePerRev".

Note: In order to make use of this macro, your cells must reverse two or more times. If you would like to apply this type of analysis to a *frz* mutant or other strain that reverses infrequently, you will need to make long movies in order to capture at least two reversal events. For example, *frz* mutants reverse once every 2 hrs, so you will need to make several 2-3 hour long movies.

To run the macro "TimePerRev":

1. Run normal macro. (needed to generate reversal frequency data)
2. Select Tools>Macro>Macros…, Select "TimePerRev" and click "Run"
3. Repeat steps 1 and 2 if multiple datasets are to be analyzed
4. Here is the resulting output in the spreadsheet "TimePerRev.":

    Column A: the name of the movie analyzed
    Column B: Cell number
    Column C: The time per reversals (in seconds)
    Column D: The length of the pause in seconds
    Column E-G: summary of the data (average for multiple datasets is in the fourth row)

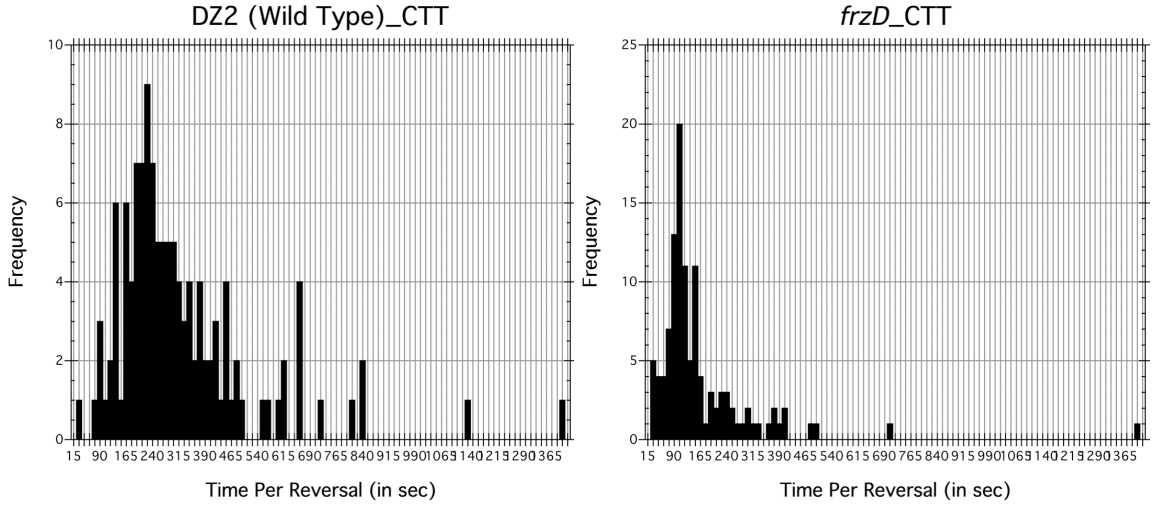


Figure 5: This was generated by plotting the data in column C of sheet "TimePerRev" as a histogram. Here you can see that the wild type strain DZ2 has a broader distribution of reversal frequencies than the hyper-reversing *frzD* strain.

2. Analysis of Pauses

The main program discards/ignores instances where cells have paused to minimize errors. However analysis of the pauses may yield useful information. Pauses may reflect failed attempts at reversing and frequent pauses may indicate an overactive chemotaxis pathway. Or a long pause before reversing may reflect an inefficient chemotaxis pathway or defects in the motility apparatus. Figure 6 below shows the results of the PausePerRev macro for the DZ2 and *frzD* strains.

To run the macro "PausePerRev":

1. Run normal macro. (needed to generate pause and reversal data)
2. Select Tools>Macro>Macros…, select "PausePerRev" and click "Run"
3. Repeat steps 1 and 2 if multiple datasets are to be analyzed
4. Here is the resulting output in the spreadsheet "PausePerRev."

Column A: the name of the movie analyzed
Column B: Cell number
Column C: The frame number where the pause occurred
Column D: The length of the pause in seconds
Column E: If "Y" (Yes) the cell reversed after pausing, if "N" (No) the cell paused without reversing
Column G-M: summary of the data:
Column G: name of the movie. The average of multiple movies is reported in row 4
Column H-I: The summary for cases where the cell reversed after pausing (a "Y" reported in column E)
Column J-K: The summary of cases where the cell did not reverse after pausing (a "N" reported in column E)
Columns L-M: The summary of all pauses regardless of reversals

4. To plot the pauses before reversals as in Figure 6, you will need to run the macro "edit_PausePerRev", which deletes the cases where a cell paused but did not reverse (deletes the rows with an "N" in Column E). The macro also adds extra rows corresponding to the number of times the cell reversed but did not pause. In these cases the pause time is not zero, but is less than the frame rate of the movie. For example, the frame for the data in Figure D was 15 seconds per frame, so from the histogram we would say that the cells are capable of reversing in less that 15 seconds.

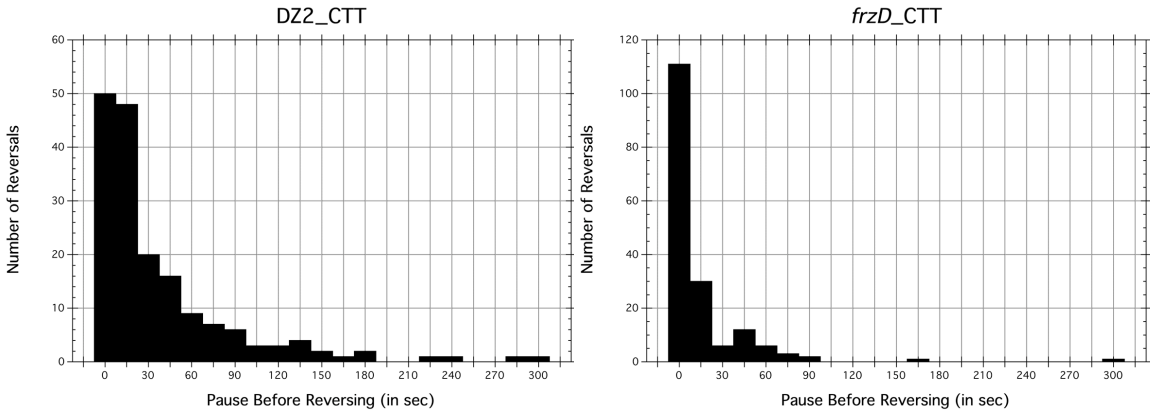


Figure 6: A histogram generated by the PausePerRev and edit_PausePerRev macros. Note that Myxo is able to execute a reversal event in less than 15 seconds and that the wild type (DZ2) pauses before reversing more often than the hyper-reversing *frzD* strain.

3. Plot trajectories of your cells

The macro "Transpose" recalculates the x, y coordinates of each cell so that the initial trajectory of each cell is pointing along the x-axis. The new x,y coordinates are placed in columns N and O in the sheet "raw." You can use these new coordinates to generate a graph as shown below.

Note: The normal Motility Macro can be run independently of the Transpose macro. Transpose does not alter any of the original data and places the new data in Columns M through O in the sheet "raw", which can then be copied and pasted in a new sheet.

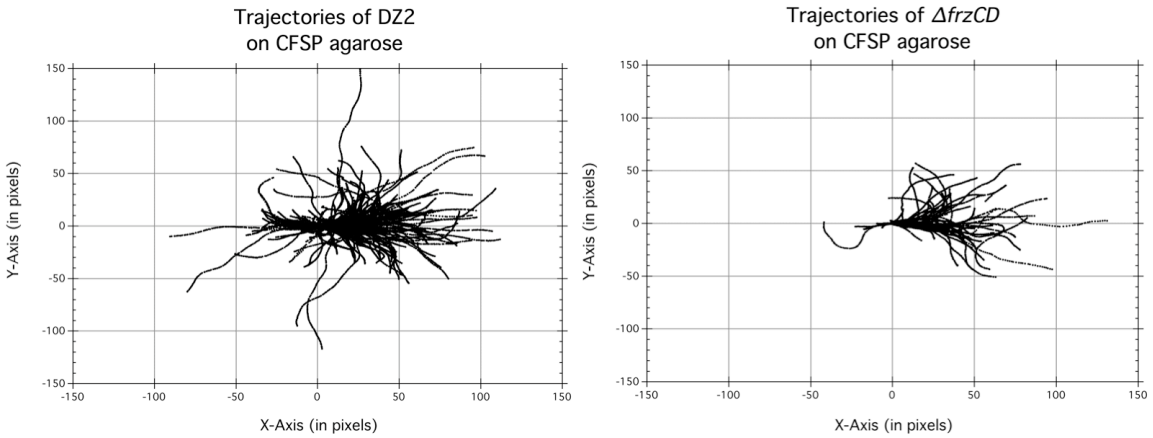


Figure 7: The trajectories of many cells of the wild type (DZ2) and the *ΔfrzCD* strains. The data was generated from the "Transpose" macro. Note that the wild type (DZ2) strain wanders much more than the *frz* mutant. Also note that the *frz* mutant only moves in one direction (to the right).

To run the macro "Transpose":

1. Paste your data in sheet "raw"
   (Note: all you need is the data in columns A through E as described previously)
2. Select Tools>Macro>Macros… from the menu
3. Select "Transpose" from the list of macros
4. Click "Run"
5. The results will be in columns M. N and O in the sheet "raw."

Acknowledgements

This macro would not have been possible without many productive discussions with Roy Welch and Hera Vlamakis. Roy wrote a similar macro which I refer to as “version 1.0”. I borrowed many of the concepts and ideas used in Roy’s macro to write version 2.0. Roy was also a valuable resource for the agar slide apparatus and filming techniques used for my movies. See (Welch R, Kaiser D (2001) PNAS 98(26) pg 1407-12) for details of Roy’s macro and filming techniques. I would also like to thank Hera Vlamakis for many helpful discussions and teaching me to use Microsoft Excel.