Spying on spirilla: Tracking diffusion in the presence of spiral bacteria

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Abstract: Spiral bacteria are commonly found in the environment, especially in viscous fluids. Their spiral shape should allow them to remain motile under conditions where other non-spiral bacteria are not. Since spiral bacteria may, at times, be the only motile cells in a viscous environment, we were interested in studying whether or not spiral bacteria are capable of influencing the dispersion of particles in their environments. We isolated two very thin (< 0.22 μm in diameter) spiral bacteria from School Street Marsh in Woods Hole, MA to investigate this idea. We used microscopy and bead tracking to monitor particle diffusion in the presence and absence of bacteria, and we found that the presence of our isolated bacterium does not significantly change the displacement of 0.2 μm diameter beads in low-viscosity fluid. Further experiments are needed to analyze how fluid viscosity, bead size, cell density, and chemotaxis may play a role in this interaction.

Introduction: Spiral bacteria can be found and isolated from many different environments. Their distinctive shapes, while fun to simply observe under a microscope, are particularly interesting in the context of bacterial motility. It has been shown that a number of spiral bacteria are able to swim through viscous fluids that would prevent motility of non-spiral flagellated bacteria[1]. This implies that spiral bacteria are able to live in and gather resources from environments in which other bacteria cannot. As the spiral bacteria may be the only motile components of an otherwise stationary, viscous environment, we were curious to know if spiral bacteria can influence the diffusion of particles that they come into contact with. Furthermore, if diffusion is influenced by spiral bacteria, could the survival and growth of other organisms be dependent on these bacteria?

Bacterial influence on particle diffusion has been quantified in previous studies, and a phenomenon called superdiffusion has been observed[2]. Essentially, it has been shown that motile bacteria can increase the diffusion of freely-diffusing particles beyond what would be expected based on the temperature and viscosity of the medium. However, we have yet to encounter any similar studies that have used spiral bacteria. Studying these bacteria is particularly interesting because they live at very low Reynolds numbers where viscous forces dominate, and it is generally assumed that these bacteria burrow through their environments without significantly disturbing the surrounding fluid.

Although any spiral bacterium would be interesting to study, we chose to focus on a very thin bacterium in the Oceanospirillaceae family that was isolated from School Street Marsh in Woods Hole, MA. We are working under the assumption that this bacterium is uniquely suited for motility through thick substances, potentially with very small pore sizes, which would prevent motility of other microorganisms. We have not tested this assumption beyond noting that this bacterium is able to move through a 0.22 μm filter and can burrow into agar plates that are 1.0% agar and below.

Methods:

Bacteria isolation: A sediment sample was collected from the edge of School Street Marsh in Woods Hole, MA and then a same-day isolation procedure was set up. Isolation was achieved by lightly wetting a teaspoon-sized scoop of sediment with 1X Fresh Water base and then spreading this mixture onto a 0.22 µm filter disk (approx. 4 cm in diameter) that was centered on an Aquatic Thin Bacteria agar plate. No sediment was placed directly onto the plate or near the edges of the filter disk. This plate was incubated at room temperature for 5 hours and then the filter and sediment were aseptically peeled away from the plate and discarded. After about two days, small colonies began to appear on the plate, many of which contained thin, spiral-shaped bacteria. Isolation was completed by re-streaking individual colonies onto fresh aquatic thin bacteria agar plates and incubating at room temperature. Colonies take about 5 days to grow large enough to work with comfortably. An Oceanospirillaceae isolated in this manner was used for further study (15BS1). It was also routinely grown in liquid Sea Water Complete (SWC) medium and on 0.75% agar SWC plates. All bacteria were grown at room temperature, either on plates on the benchtop or shaking in liquid.

Isolation and culture medium: 100X Fresh Water base contains 1711 mM NaCl, 197 mM $MgCl_2$, 68 mM $CaCl_2$, and 671 mM KCl.

1X Seawater base contains 342.2 mM NaCl, 14.8 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 6.71 mM KCl.

Sea Water Complete medium: 1L of 1X Seawater base with the following additions: 5g bacto tryptone, 1 g yeast extract, and 3 mL glycerol. This solution was brought to pH 7 and then autoclaved. For 0.75% agar plates, 7.5 g of bacto agar was added to 1L of SWC medium before autoclaving.

Aquatic Thin Bacteria Medium: per 1L of medium, add 10 mL of 100X Fresh Water base, 5 mL of 1 M MOPS pH 7.2, 0.5 g yeast extract, 0.5 g tryptone, 1 mM maltose, 1 mM sodium succinate, and 12 g of bacto agar. Autoclaved.

Genetic analysis: 16s analysis was performed on a colony of 15BS1. The colony was boiled for 10 min in alkaline PEG200 (ALP), spun down, and then 3 µL of the supernatant was used as a template for a PCR. Promega GoTaq® G2 Hot Start Green Master Mix was used for PCR with 8F and 1391R primers. The tree was assembled using RDP[3].

Cell-shape analysis: Images from phase contrast microscopy movies were converted into binary using Fiji and then loaded into Celltool to analyze the overall shapes[4,5]. The analysis mirrored the tutorial on the Pincus lab website except for changes in the image dimensions.

Microscopy: Images and movies were taken on a Carl Zeiss Axio Observer.Z1 inverted microscope using a 100X oil objective. Bacterial samples were taken from the top of a shaken liquid culture to obtain those with the greatest motility. Fluorescent 0.2 µm beads were washed ≥ 5 times in SWC medium and then methyl cellulose was added to a final concentration of ~0.02% (to keep beads suspended) before the beads were mixed with the bacteria at appropriate ratios (~300 beads per field of view at 100X magnification). Samples were placed into a MatTek dish with a cover glass bottom and another piece of cover glass was placed on top such that most of the beads were in the same plane. The MatTek dish was used to keep flow to a minimum. Movies were 1.5 min long with frames taken approximately every 0.5 seconds. Both phase contrast and fluorescence (Lucifer yellow) movies were recorded. An Axiocam 503 mono camera was used to capture, an HXP 120 V lamp was used for fluorescence, and Zen2 software was used to run the microscope and record images/videos.

Microscopy analysis: Movies were first analyzed using the TrackMate plugin for Fiji[4]. Each bead was first identified using the following processes/criteria: LoG detector, estimated blob diameter = 3.0 micron, threshold = 0.0, use median filter, no quality filter on bead identification. Hyperstack displayer was then used followed by a simple LAP tracker with the following criteria: linking max distance = 5.0, gap-closing distance = 5.0, gap-closing max frame gap = 5. Once tracks were identified, they were filtered by: track displacement = above 0.3, duration = above 7.16, longest gap = below 2.0, and number of gaps = below 6.0. Once tracks were identified, the tracking information was imported into Matlab to subtract residual flow from all of the tracks, calculate the mean square displacement for each video, calculate coefficients of diffusion, and then plot all of the data (Supplemental).

Results:

16s characterization: We first set out to characterize the thin spiral bacterium that we had isolated. Sequencing of the 16S from 15BS1 and another isolate (15BS2) showed that we had isolated two Oceanospirillaceae and that the closest cultured relatives were *Marinomonas* species. Using nBLAST, we found that both new isolates share 96% identity with the marine bacterium TW-1 and *Marinomonas* species SBI22[6]. Coverage is 94% and 93% for the two isolates, respectively, against both of the related BLAST hits (E value = 0.0). Figure 1 is a phylogenetic tree of our two isolates, five of the most closely-related species, and a small selection of other *Marinomonas* species and spirochetes. We chose *Aliivibrio fischeri* to be an outgroup. Our two isolates are more closely related to each other than to any other cultured species, and due to the relatively low sequence identity to other cultured species, we are sequencing 15BS1. This will allow us to gain a greater understanding of its metabolic potential. All future experiments were done using 15BS1.

Cell shape: We completed a preliminary analysis of the shape of 15BS1 grown in SWC liquid medium. Principal component analysis shows that the greatest difference in cell shape is in cell length and, to a lesser degree, in the degree of curvature at the ends of the bacteria (Figure 2). Overall, the amplitude and pitch of the spiral cells appear to remain consistent across cells. We did not obtain great enough resolution to precisely measure the diameter of these cells, but given their ability to cross a 0.22 μm filter, they are probably less than 0.22 μm in diameter. Length appears to vary from about 2 to 7 μm.

Bead-diffusion: To determine whether or not 15BS1 is able to influence the diffusion of particles in its environment, we recorded the movement of 0.2 μm fluorescent beads in the presence and absence of bacteria. Phase contrast movies were also recorded to measure the swimming speed of these bacteria (approximately 30 μm per second). For speed analysis, bacteria were tracked manually and the mean speed was plotted across all of the tracks that were recorded (Figure 3). For the bead-diffusion movies, we only recorded videos in the fluorescence channel. Once movies were recorded, we used the TrackMate plugin in Fiji to identify the beads and then assemble the bead positions into tracks so that overall bead diffusion could be monitored. Since there is still some residual fluid flow due to evaporation, even within sealed MatTek dishes, we performed a flow-subtraction from each of movies such that, at each timepoint, the average displacement in the x and y directions for all of the particles was subtracted from each particle (Supplemental). In this way, only diffusion is being analyzed and fluid flow is ignored. Figure 4A,B shows the difference in tracking for one of the movies before and after flow subtraction.

Once flow is subtracted, we calculated the mean square displacement (MSD) of the particles in each of the movies (Figure 4C). We are not sure why the linear MSD versus time plots start to break down beyond approximately 30 seconds of video, but we chose to further analyze only the data before 30 seconds. Since we are interested in diffusion, and the diffusion coefficient is proportional to the slope of MSD versus time, we next calculated the slope of the MSD versus time lines in the presence and absence of bacteria.

We find that the mean slope of the MSD versus time line in the presence of bacteria is 2.9 and is 1.9 without. However, using a two-sample t-test, we find that these means are not significantly different at the 5% significance level. Thus, there is not a significant difference in 0.2 μm particle diffusion in the presence and absence of 15BS1.

Discussion: This is just a preliminary analysis of diffusion in the presence of spiral bacteria, and although the results do not show a significant difference in particle diffusion in the presence of bacteria, future experiments may be able to differentiate between these two conditions. For example, in Wu *et al*., the beads were 10 μm in diameter and cell density was much greater than what we used in our experiments[2]. Larger beads may experience greater overall bacterial forces simply because multiple bacteria may be able to act on a single bead, and increasing the overall cell density has been shown to increase the observed bead diffusion[2]. On the other hand, it could also be interesting to dramatically decrease the particle size by measuring diffusion of a dye or other small molecule. Using dye, we could measure the flow produced by the bacteria as they swim. Thus, although we could not detect a significant dispersal difference for particles that are a similar diameter as the bacteria, we could potentially detect a difference with very large or very small particles instead. Comparing superdiffusion of very small versus very large particles may be able to differentiate between superdiffusion caused by bacteria-induced fluid flow (dye) or from collisions (large beads).

We have not had an opportunity to measure diffusion under different viscosity conditions, which is what we initially intended to do. Our microscopy was done using a medium with a viscosity at or near the level of water, but our experiments would have been much more relevant under high viscosity conditions, as 15BS1 likely lives in high viscosity conditions in the environment. Increasing viscosity would also reduce Brownian motion, which could enhance our detection of the dispersion signal over the random diffusion background. Extensions of this study could also include analyzing dispersal during chemotaxis of the bacteria. Unfortunately, since we cannot completely remove fluid flow from our samples, we must mathematically remove flow during analysis. Because of this flow subtraction, if we were to measure dispersion under chemotaxis conditions, we would likely be subtracting out the effects we were trying to measure because they would appear as simple fluid flow. Indeed, we may already be having that problem, as we observe significant aerotaxis in 15BS1, and some of the fluid flow we subtracted could have been caused by behavior instead of abiotic evaporation.

Although we were unable to show a significant difference in 0.2 μm bead dispersal in our experiments, we are not ready to discount the idea that spiral bacteria are able to enhance particle dispersal in their environments. If there are conditions under which this idea is true, it could reveal important roles for spiral bacteria. Since other bacteria would presumably be less

motile under the viscous conditions that these bacteria grow in, less-motile bacteria could be dependent on the presence of spiral bacteria for nutrient acquisition and growth.

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References:

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Figures:

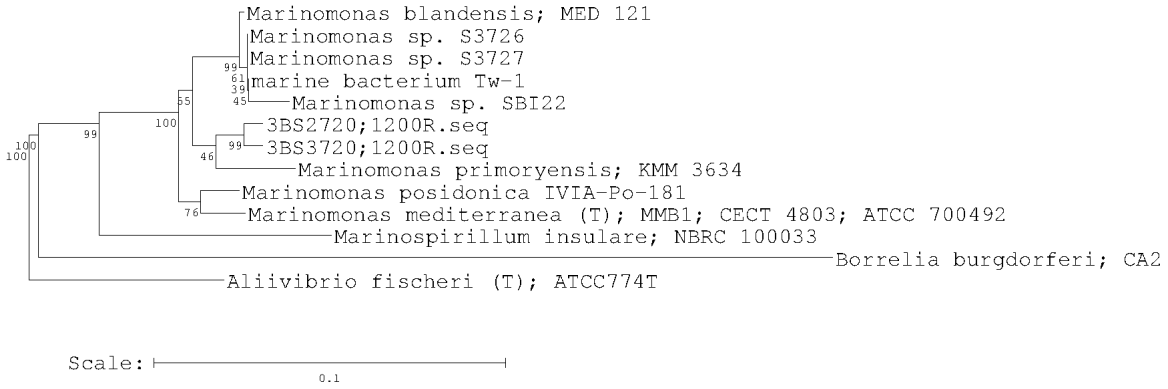

Figure 1 | Phylogenetic tree for the two new isolates 3BS2720 and 3BS3720[3]. 3BS2720 is the same strain as 15BS1. This tree was constructed in RDP using five of the isolates that are the closest-related to the two new isolates. Additional *Marinomas* strains were added to determine where the new isolates fall within the *Marinomonas* genus. *Aliivibrio fischeri* was used as an outgroup. Bootstrap values are presented.

Figure 2 | Variance in cell shape for motile 15BS1 using Celltool[5]. Images were taken with a 100x oil objective using phase contrast on an inverted Zeiss Observer.Z1 microscope. Cells of 15BS1 that were moving slow enough to capture their spiral shapes were isolated from the movies as single frames and then converted to binary using Fiji[4]. These images were then loaded and analyzed in Celltool following the tutorial on the Pincus lab website[5]. The scale bar is 1 μm.

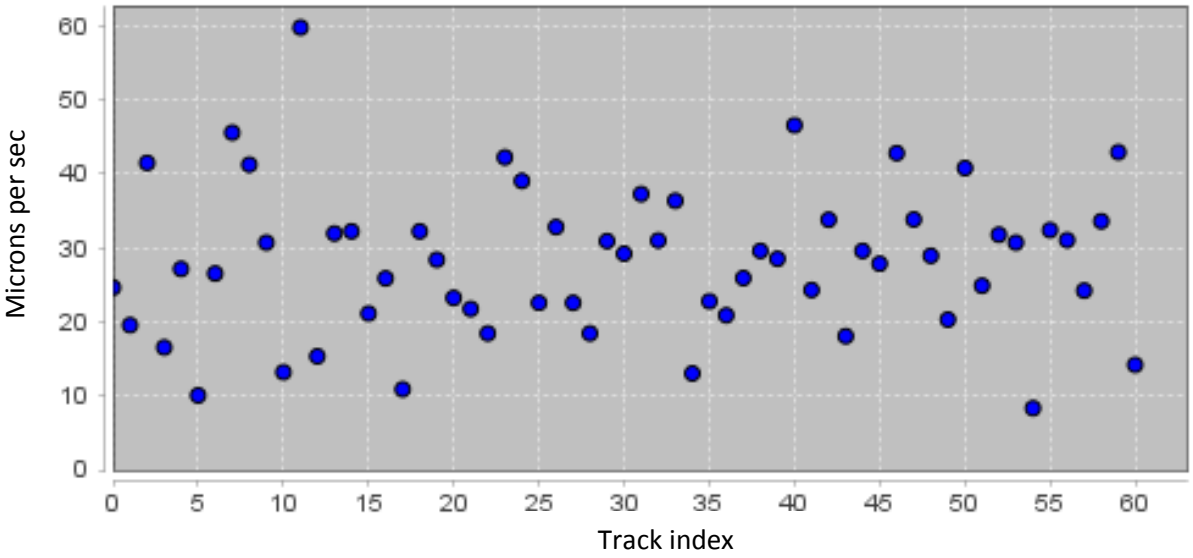

Figure 3 | Median velocity of actively motile 15BS1 in low viscosity medium. Movies were taken with a 100x oil objective using phase contrast on an inverted Zeiss Observer.Z1 microscope. Tracking was completed using the TrackMate plugin in Fiji[4]. Tracks were recorded manually.

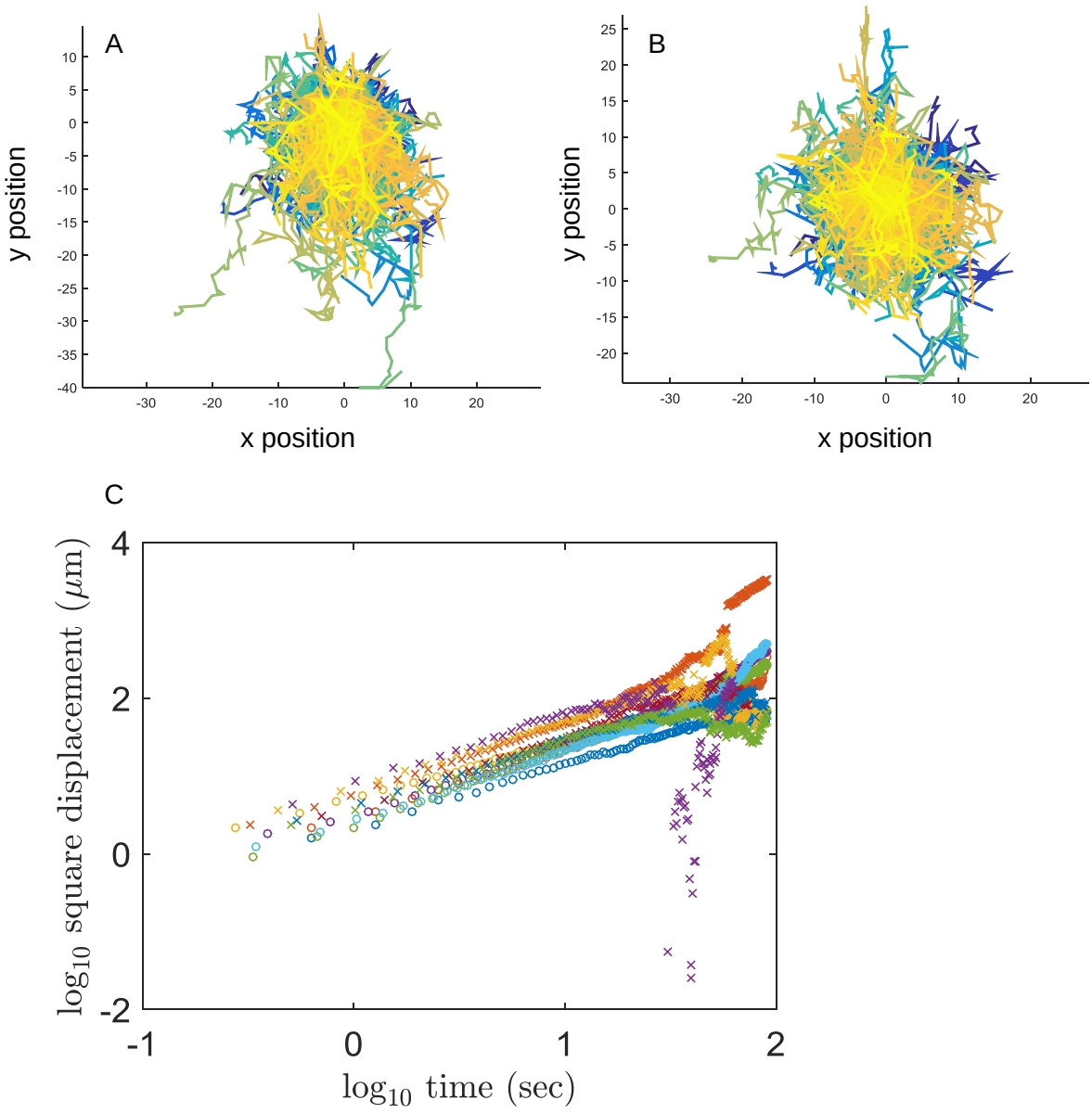

Figure 4 | Bead tracking and mean square displacement analysis. Movies were taken with a 100x oil objective using Lucifer yellow fluorescence detection on an inverted Zeiss Observer.Z1 microscope. Tracking was completed using the TrackMate plugin in Fiji[4]. A) Tracks obtained from a single representative video of the spirilla plus beads condition before flow is removed. B) Tracks obtained from the same video from part A but after flow has been removed using Matlab. C) Mean square displacement versus time for the beads only (o) and the beads plus spirilla (x) conditions. Further analysis was completed from time 0 to 30 seconds. The slopes of the lines for the two conditions are not significantly different.

Supplemental: Matlab scripts and functions used:

To re-format the TrackMate output for further processing:

```
function out_matrix = preTrack(xlsx_file)
input = xlsread(xlsx_file);
mid_matrix = input(:,[9, 10, 8]);
counter = 1;
ids = ones(length(input), 1);
for i = (2:length(input)-1)
    if input(i, 8) < input(i-1, 8)
        counter = counter +1;
    else counter = counter;
    end
    ids(i) = counter;
end
ids(length(input)) = ids(length(input)-1);
out_matrix = [mid_matrix ids];
```

To remove flow from TrackMate tracking (Mikhail Tikhonov):

```
function [flow, newData] = subtractFlow_2(data)
% input: 4-column format
% x y t id
x = data(:,1);
y = data(:,2);
t = data(:,3)+1;
id = data(:,4);

maxT = max(t);

% coordinates of a hypothetical non-diffusing particle carried around by
% flow only (to be subtracted from all the traces)
flowX = zeros(1,maxT);
flowY = zeros(1,maxT);

for t0= 1:maxT-1
    % find all particles seen at both time t0 and to+1
    % at time t0
    id_atThisT = unique(id(t==t0));
    % at time t0+1
    id_atNextT = unique(id(t==t0+1));
    ofInterest = intersect(id_atThisT, id_atNextT);
    % calculate mean displacement of all such particles

    % where in the table are the events "particle of interest was observed at
time t0"?
    % criterion: t==t0, and id in the list of interest
    ofInterest_thisT = t==t0 & (ismember(id, ofInterest));
    ofInterest_nextT = t==(t0+1) & (ismember(id, ofInterest));

    dX = mean(x(ofInterest_nextT)-x(ofInterest_thisT));
    dY = mean(y(ofInterest_nextT)-y(ofInterest_thisT));
```

```
    % declare that this mean displacement was cused by flow
    % => use this to construct flowX and flowY at this time t0
    flowX(t0+1) = flowX(t0)+dX;
    flowY(t0+1) = flowY(t0)+dY;
end
flow = [flowX; flowY];
% now tht we determined the flow, adjust the table accordingly
for i = 2:maxT

x(t==i) = x(t==i) - flowX(i)';
y(t==i) = y(t==i) - flowY(i)';

newData = [x y t id];
end
```

To measure and plot bead position and mean square displacement (Alex Petroff):

```
function [bead,time,msd]=track_diff(results,dx,dt)
% dx=pixel size
% dt=frame rate of camera (in seconds per frame)

x=results(:,1);
y=results(:,2);
t=results(:,3);
id=results(:,4);

ids=unique(results(:,4));
bead=cell(length(ids),1);

%% make a cell the that contains the times, positions, and displacments of
each particle
for i=1:length(ids);
    x0=x(id==ids(i));
    y0=y(id==ids(i));
    t0=t(id==ids(i));
    [t0,I]=sort(t0);
    x0=x0(I);
    y0=y0(I);

    % time since bead was first seen
    bead{i}.time=(t0-t0(1))*dt;

    % bead position
    bead{i}.pos.x=x0;
    bead{i}.pos.y=y0;

    % squared bead displacment simce first frame
    bead{i}.dis=dx*dx*((x0-x0(1)).^2 + (y0-y0(1)).^2);
end

%% collect all of the data
times=[];
disps=[];
```

```
for i=1:length(ids)
    times=[times;bead{i}.time];
    disps=[disps;bead{i}.dis];
end

%% find the mean square displacement as a function of time by avereging over
trajectories

time=unique(times);
msd=ones(length(time),1);
for i=1:length(time);
    t_=time(i);
    msd(i)=mean(disps(times==t_));
end

figure
plot(log10(time),log10(msd),'o','markerfacecolor','b','markersize',5)
hold on
% plot(log10(times),log10(disps),'.r')
fs=18;
set(gca,'fontsize',fs)
xlabel('$\log_{10}$ time (sec)','interpreter','latex','fontsize',fs)
ylabel('$\log_{10}$ square displacement
($\mu$m)','interpreter','latex','fontsize',fs)

figure
cm=colormap;
vec=linspace(1,length(ids),size(cm,1));
rr=interp1(vec,cm(:,1)',1:length(ids));
gg=interp1(vec,cm(:,2)',1:length(ids));
bb=interp1(vec,cm(:,3)',1:length(ids));

hold on
for i=1:length(ids)
    plot(bead{i}.pos.x-bead{i}.pos.x(1),bead{i}.pos.y-
bead{i}.pos.y(1),'color',[rr(i) gg(i) bb(i)],'linewidth',2);
end
axis equal
```