Supplementary documentation.

1.Microscope acquisition techniques

Despite the strong potency of white light microscopy, its main characteristic is the lack of contrast. The light field is the main and simplest technique in it, contrast is caused by the attenuation of transmitted light. It is useful for samples that have intrinsic color or when staining is used. Other techniques have been developed to replace its deficiencies such as dark field lighting, in this case the contrast comes from the light scattered by the illuminated sample. This is the same principle that allows us to see stars at night or dust particles in a ray of light when we enter a dark room. That is, in the dark field technique, samples appear as a luminous object on a dark background, which is particularly suitable for samples with low contrast (for example to study physiological processes such as mitosis or cell migration). However, unless the medium is extremely thin, the dark field mode can distort details. Phase contrast lighting is also a common white light microscopy technique; it is based on interference of different light path lengths through a sample. The heterogeneous cellular components absorb light differently and cause small phase variations in the luminous. In uncolored cells and tissues, the low contrast is enhanced and accentuated by transforming phase differences into luminous intensity differences. In this case, details in the sample have a halo effect around them which can deteriorate image resolution. Nonetheless, phase contrast is useful for live tissues and '*in-vivo'* experiments. [37]

Fluorescence imaging is an invaluable tool for scientific research since it tolerates high levels of sensitivity and microscopic resolution. It has its drawbacks such as the limited number of available fluorescent channels in microscopes or the overlapping excitation and emission spectra of the stains. In addition, phototoxicity is much higher in the fluorescence microscope because fluorophores needed to produce fluorescence which are unstable compounds that can damage the chemical structure and biological function of proximal particles [38]. In addition, photobleaching occurs, it is a chemical damage in the fluorescent molecules due to the electrons excited during fluorescence, this will cause the loss of the ability to fluoresce in the illumination process over time, changing the intensity of the image for the same structure. Fluorescence microscopy is used in marking molecules in cells and tissues for characterization and identification, study of normal and pathological cells or in immunological studies.

In all the methods described above, the sample is illuminated completely, and the image is only focused on a specific plane at a time, unfocused planes above and below inducing artifacts in the captured image. Multiple techniques have been developed with the aim of being able to project all the planes to only one without losing information[39]. As stated before, these limitations have been already overcome with confocal microscopy and it is possible to reconstruct a 3D image with all planes focused. With this acquisition technique, samples must express fluorescence, thus presenting the aforementioned drawbacks. Additionally, since the required technology is much more advanced, it is very expensive and the image capture requires long time. Besides, files generated are much larger and the post-processing of the images is more complex. Figure 3 shows some examples of images taken with different optical microscopy techniques.

2.Pseudo code for Cell association methods.

CellAsociation (Correlation)

1. Get coordinates of the cell in last frame
2. Get template from previous image
3. Get search window in current image
4. Calculate the correlation between template and search image.
5. Find max of correlation and stablish new cell centroid

CellAsociation (SegCorr)

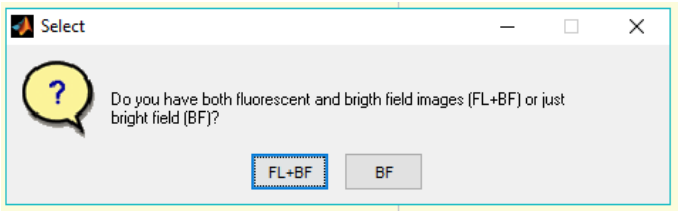
1. Get coordinates of the cell in last frame
2. Get template image from previous frame and process it doing gradient, dilation, reconstruction and maximal region.
3. Get the marker doing the segmentation of template image
4. Get search image from actual frame and process it doing gradient, dilation, reconstruction (with marker) and maximal region
5. Calculate the correlation between template and search image
6. Find max of correlation and stablish new centroid.

CellAsociation (SegCorrMC)

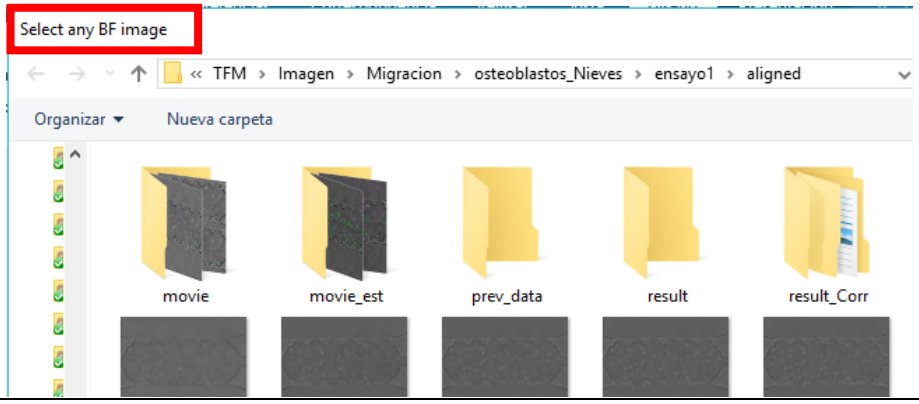
1. Get coordinates of the cell in last frame
2. Get template image from previous frame and process it doing gradient, dilation, reconstruction and maximal region.
3. Get the marker doing the segmentation of template image
4. Get search image from actual frame and process it doing gradient, dilation, reconstruction (with marker) and maximal region
5. Calculate the correlation between template and search image
6. Find max of correlation and stablish new centroid.

3.Example of migration analysis in Matlab platform.

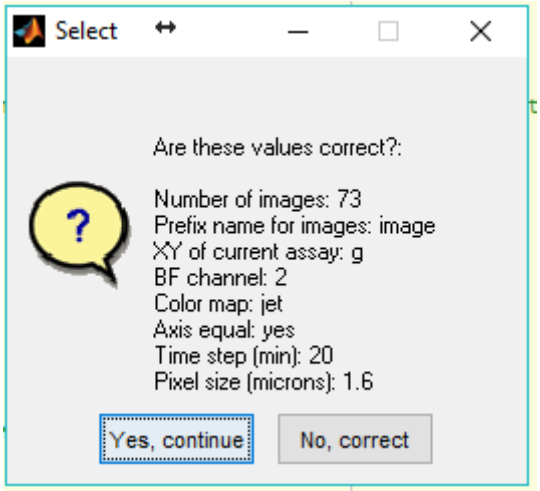
1. Choose the type of assay.

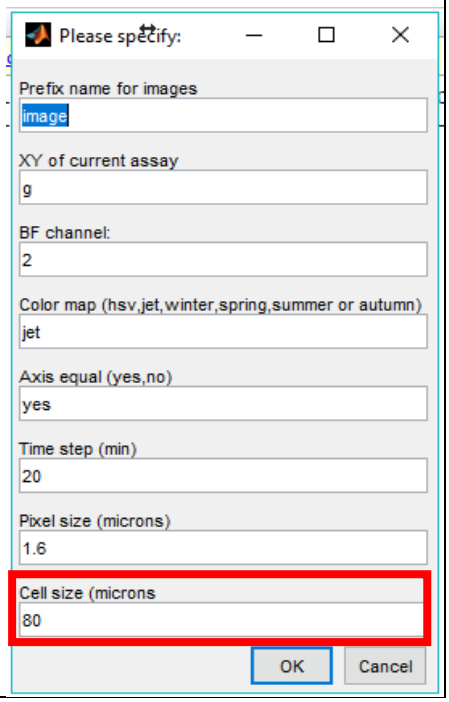


2. Select the folder where the images assays are

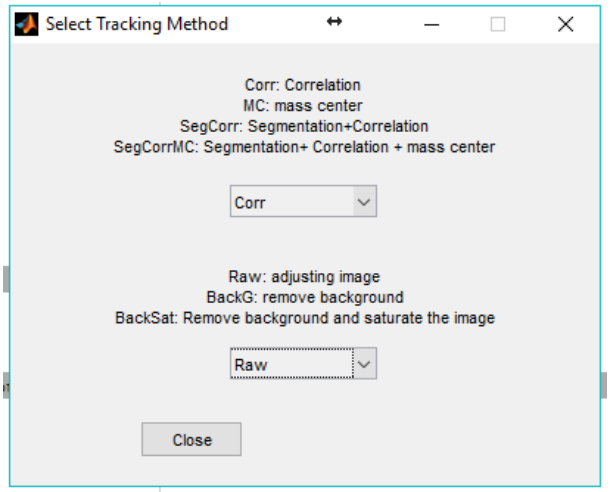


3. Confirm that all values are correct or modify them

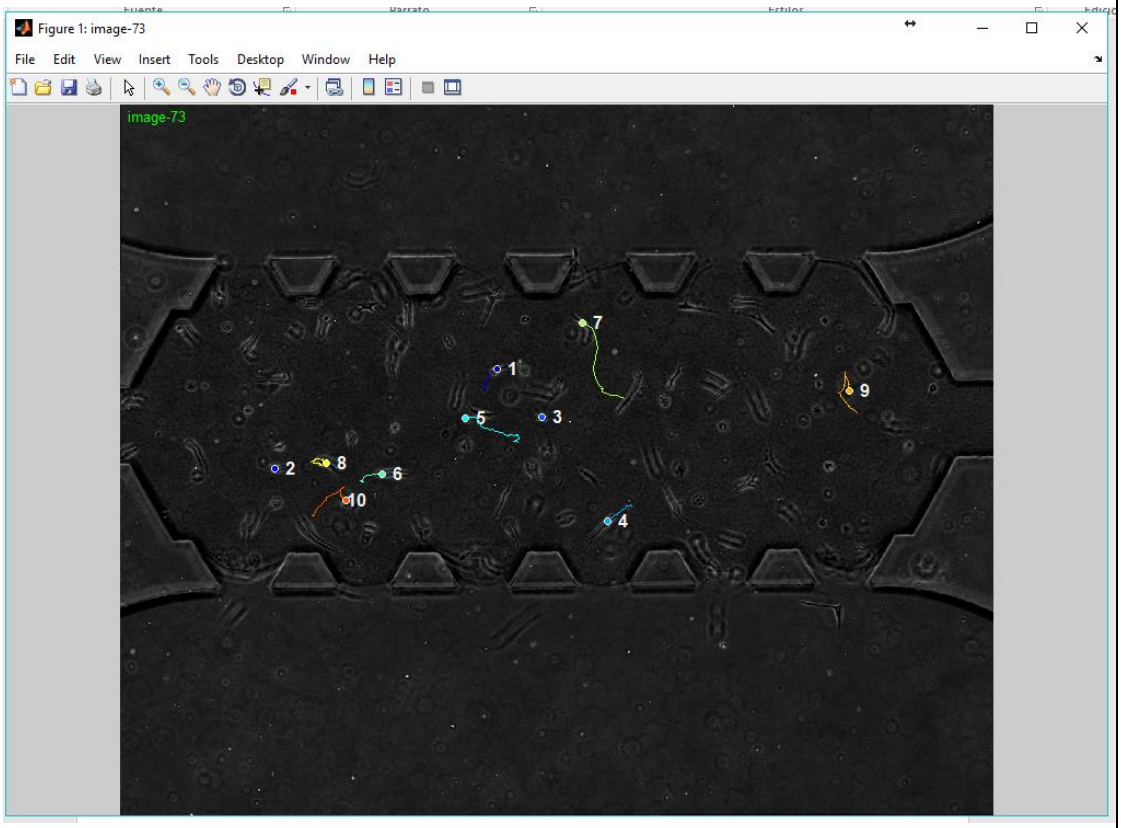




4. Select tracking and preprocessing method



5. Choose cells to analyze and get trajectories



6. Get results: Some representative graphs are plotted in order to analyze the migration. These graphs are represented in Figure S1 and S2

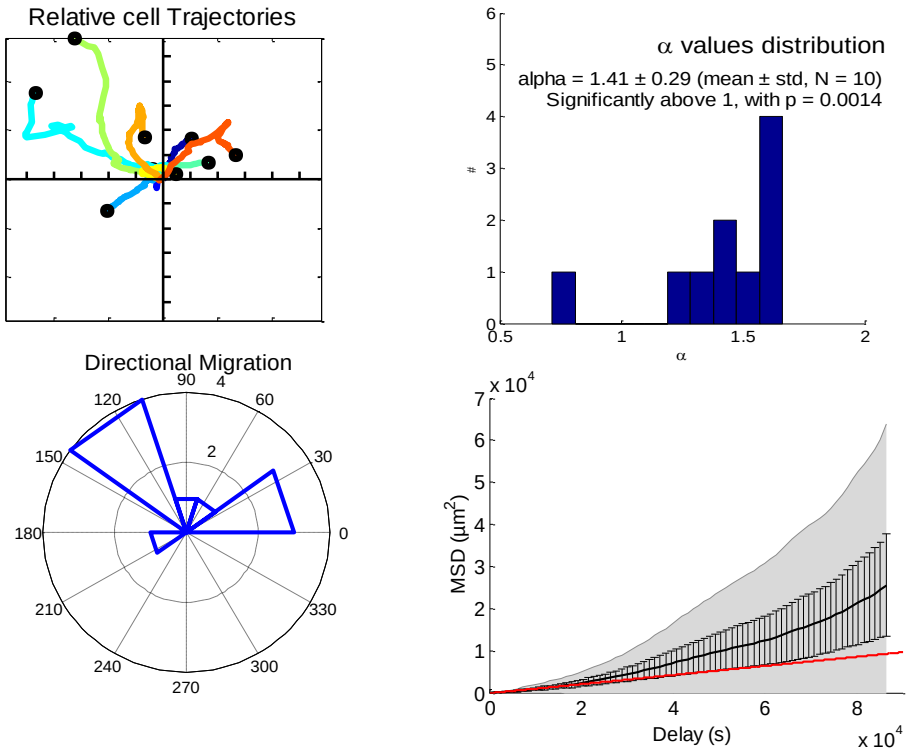


Figure S1. In the left column it is represented the trajectories of the individual cells (in the first row) and its angular histogram in the second row (it is taken into account only the first and last point of each trajectory). In right column it is represented the diffusivity coefficient (α), in the first row with a histogram distribution. In second it is represented the minimum square distance from each point of the trajectory from the initial one in comparison with the red line which represents α =1 and means the perfect linear trajectory.

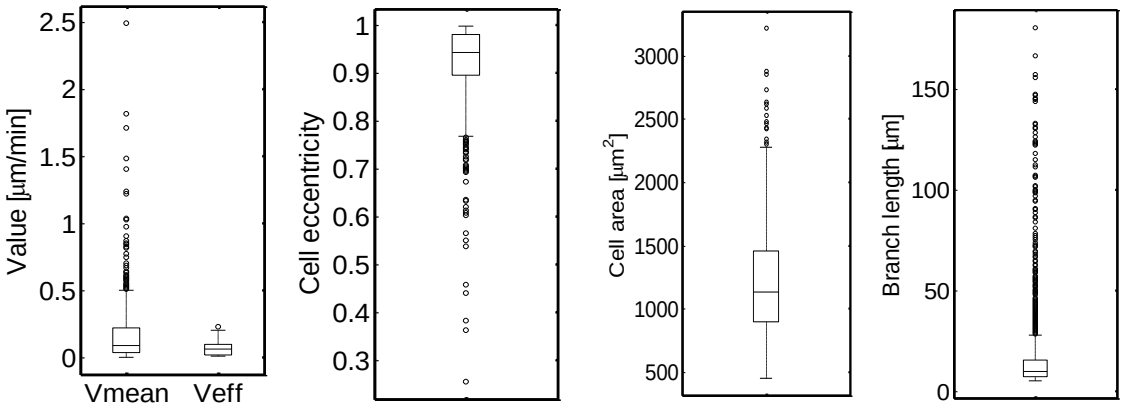


Figure S2. Boxplot of some variables extract from cell migration. The first one mean and effective speeds. The second the cell eccentricity. The third one cell area. And the las graph represents branch length.

7. If you analyze many assays with different factors it is possible to compare them and get some statistical results and comparison graphs as shown in Figures S3-S6. These results are extracted from an osteoblast migration experiment [35].

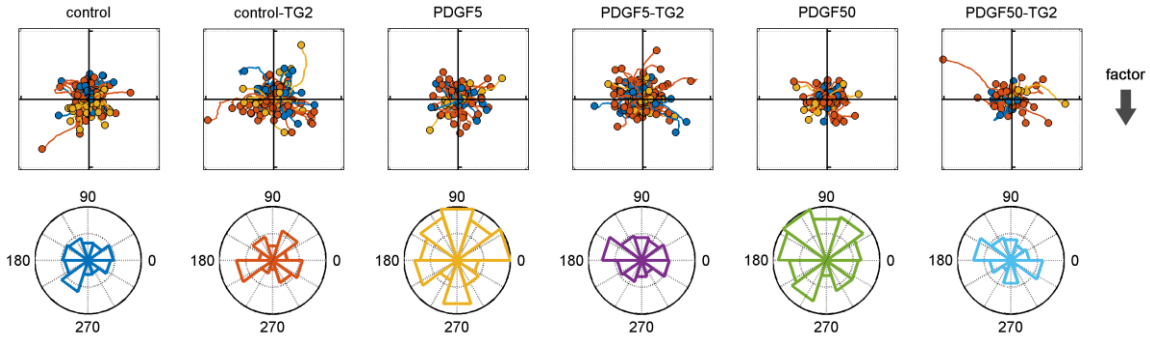


Figure S3. Trajectories of individual cells (first row) and polar histograms (second row). Histograms shows the angular distribution of the trajectories taking into account only the first and lat point of each trajectory. Black row indicates the direction of the factor gradient.

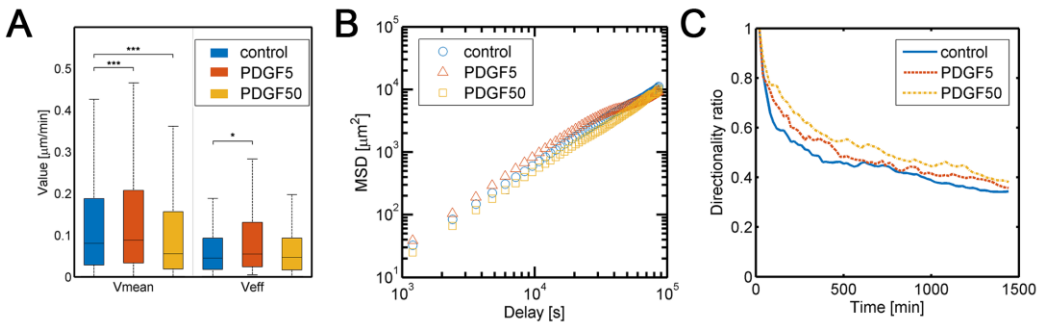


Figure S4. A) Mean and effective speeds of HOB cells, B) mean squared displacement (MSD) of tracked trajectories and C) directionality ratio of 4mg/ml of collagen with 0 (control, blue), 5 (orange) and 50 yellow mg/ml PDGF-BB.

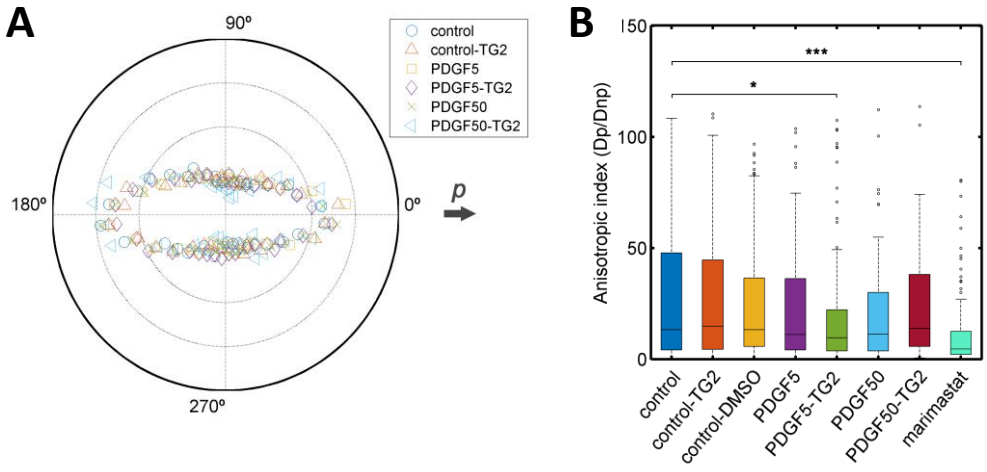


*Figure S5. A) Velocity magnitude polarization profile: average magnitude of cell speed, re-aligned along the primary migration direction (p) and evaluated at different orientations. If the velocity is isotropic (a true random walk) the average magnitude of cell speed is equally likely in all directions. B) Boxplot of anisotropic index calculated dividing the diffusivity coefficient in the principal direction by the non-principal direction. Each color represents one different factor. ***p<0.001; **p<0.01; *p<0.05.*

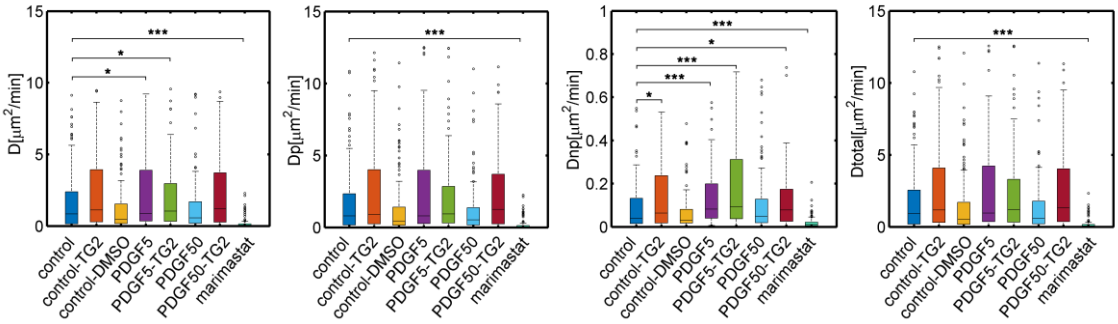


*Figure S6. First line is the estimated diffusion coefficient calculated by fitting a PRW model (D) and an APRW model (Dp and Dnp). Dtotal is the sum of Dp and Dnp and has the same meaning of D in the PRW model. Second line estimated diffusion . ***p < 0.001; **p < 0.01; *p < 0.05.*

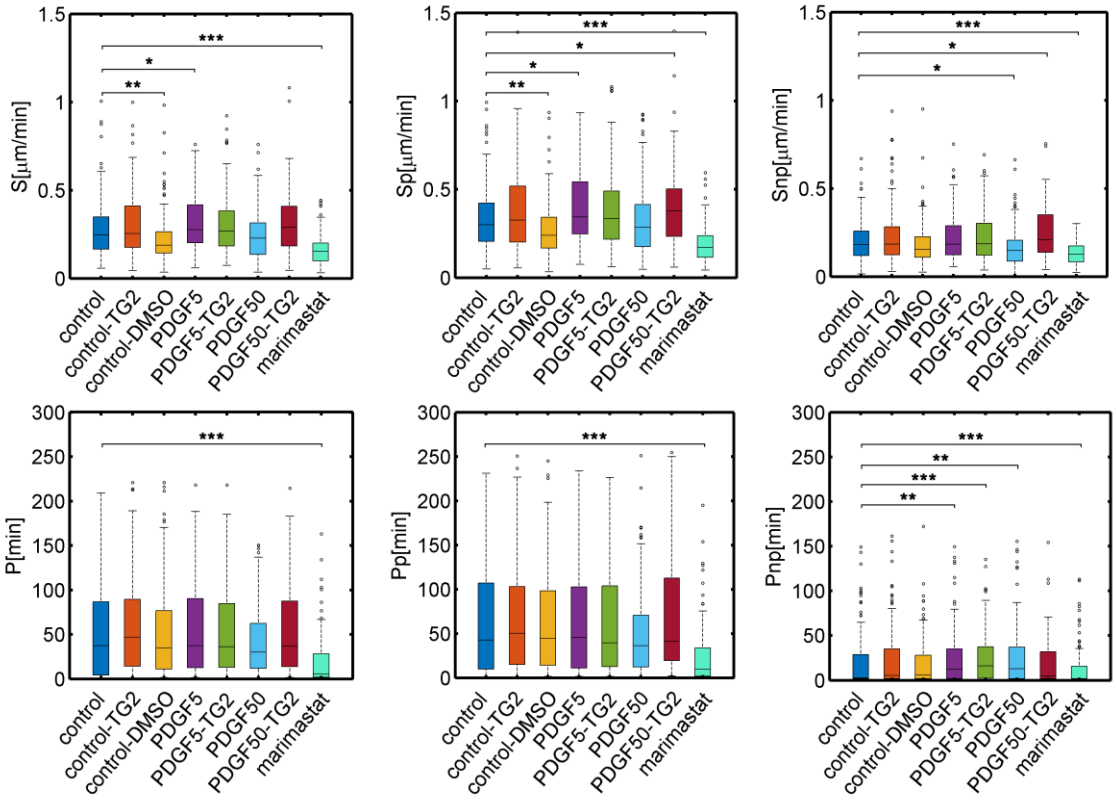


*Figure S7. First row: persistence speeds obtained by fitting a PRW model (S) and an APRW model (Sp and Snp). Second row: persistence times obtained by fitting a PRW model (P) and an APRW model (Pp and Pnp). ***p < 0.001; **p < 0.01; *p < 0.05.*

4.Tracking videos.

In the following links are 7 videos to show cell migration and tracking methods.

- Tracking 5traj XXX shows the comparative trajectories for the different methods all without any preprocessing, the legend color is as follows:

  Red: Manual tracking
  Blue: Correlation method.
  Yellow: Mass Center based method
  Green: Correlation +Segmentation method
  Cyan: Correlation+Segmentation+MassCenter based method

  Cardiac: https://youtu.be/fkdSJFBDNPw
  Epithelial: https://youtu.be/7_AC7cqgcr0
  Osteoblast: https://youtu.be/y4K9aoRUoLc
  Stem: https://youtu.be/mdHThz7uYxA
  2D: https://youtu.be/tvWTHHISeiE

- BackgroundG shows an example of osteoblast stack images with removed background https://youtu.be/eA4S079VJok

- BackgroundSat.avi shows the same assay as before (osteoblast stack) with background removed and image saturated.
  https://youtu.be/dJTxlvQvADI