

Integrative Toolkit to Analyze Cellular Signals: Forces, Motion, Morphology, and Fluorescence

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Abstract

Quantitative assessment of cellular forces and motion advanced considerably over the last four decades. These advancements provided the framework to examine insightful mechanical signaling processes in cell culture systems. However, the field currently faces three problems: lack of quality standardization of the acquired data, technical errors in data analysis and visualization, and perhaps most importantly, the technology remains largely out of reach for common cell biology laboratories. To overcome these limitations, we developed a new experimental platform - Integrative Toolkit to Analyze Cellular Signals (iTACS). iTACS consists of two components: Acquisition and Training Module (AcTrM) and Analysis and Visualization Module (AnViM). AcTrM is based on μ Manager - an NIH-ImageJ-based microscope control software - and facilitates user self-training and automation of common image acquisition protocols. AnViM is based on NIH-ImageJ and facilitates user-friendly automation of data analysis and insightful visualization of results. These experiments involve culturing adherent cells on hydrogels, imaging fiducial markers embedded in the hydrogel, and finally extracting from these images a comprehensive mechanical characterization of the cells. Currently, iTACS enables the user to analyze and track a wide array of properties, including morphology, motion, cytoskeletal forces, and fluorescence of individual cells and their neighboring region. The quality standardization issue was addressed in AcTrM with, a reference image-guided refocusing technique. The technical issues in data analysis were addressed in AnViM with a multi-pronged image segmentation procedure, a user-friendly approach to identify boundary conditions, and a novel cellular property-based data visualization. AcTrM is designed to facilitate the

straightforward transformation of basic fluorescence microscopes into experimental cell mechanics rigs, and AnViM is equipped to enable users to measure cellular mechanical signals without requiring an engineering background. iTACS will be available to the research community as an open-source suite with community-driven development capabilities.

Introduction

Commonly used optical imaging and data analysis tools employ hardware and software technologies that are nearly antiquated. The lag in translation and implementation of advances in electronic devices, computational approaches, and mathematical analysis into common experimental cell biology tools is a major constraint on the pace of growth in our knowledge of cellular physiology. Currently, cell biology researchers find molecular biology tools within the reach, but tools based on engineering principles to be out of reach. One such engineering-principles-based tool is Monolayer Stress Microscopy (MSM)^{1,2}. While MSM has been adapted and studied in various laboratories worldwide, its use is primarily confined to labs with engineering expertise^{3,4,5,6,7,8,9}.

NIH-ImageJ is one of the most popular open-source tools among cell biology researchers¹⁰. User community contribution-driven advancements have been central to its popularity^{11,12}. ImageJ has features that allow users to develop applications with a mix of an advanced programming language and simplified scripting approaches. These features facilitate users with basic programming knowledge to implement, adapt, and advance any new contribution to the software. Building on these qualities of NIH-ImageJ, we have developed the Integrative Toolkit to Analyze Cellular Signals (iTACS), which enables a low-cost integration of desired hardware and software tools to automate the measurement

of a wide variety of chemical and mechanical signals across adherent cells^{11,12}.

iTACS comprises two components: Acquisition and Training Module (AcTrM) and Analysis and Visualization Module (AnViM). AcTrM is built on μ Manager - an NIH-ImageJ-based image acquisition application - to enable users to set up time-lapse measurements of traditional optical properties and a variety of physical properties of adherent cells in multiple samples¹². AcTrM facilitates user training through concise directions included in the graphical interface. In addition, it has a novel feature of reference image-based autofocusing that is designed to facilitate real-time measurements of physical forces and enable quality standardization of the acquired data.

AnViM is built on ImageJ plugins, expedited software, and file handling scripts that enable users to quantitatively assess more than 50 properties, including cellular shape, size, orientation, speed and direction of motion, tractions exerted on the extracellular matrix (ECM), and on neighboring cells, contractile and shear moments of both individual adherent cells and their neighboring region. AnViM facilitates users to quantify the cellular physical properties without mastering the underlying technical background¹¹. Furthermore, it enables data analysis in an interactive or batch processing mode. It generates heat maps revealing spatial variation and graphs

showing the temporal variation of the properties of individual cells.

In a typical experiment, the user cultures cells on an elastic hydrogel with appropriate extracellular matrix proteins on the top surface and two types of embedded fluorescent markers. Essentially, images of these fluorescent markers before and after culturing the cells are sufficient to quantify the forces within and around individual cells^{2, 13}. AnViM maps these results onto individual cells of the adherent cluster and generates insightful images and graphs.

Protocol

NOTE: Samples examined using the iTACS platform are cells adhered to a soft substrate. The protocol for assessing mechanical and chemical signals is divided into two sequential parts: Acquisition and Training Module (AcTrM) and Analysis and Visualization Module (AnViM).

1. Acquisition and Training Module (AcTrM)

NOTE: AcTrM automates the process of data acquisition and user self-training. Before any data acquisition, prepare a soft substrate capable of providing information necessary to quantify forces that cells exert on it.

1. Hydrogel preparation

NOTE: The goal here is to prepare a 1,250 Pa shear modulus, approximately 100 μm thickness, and 22 mm diameter polyacrylamide (PAA) hydrogel.

1. Prepare polyacrylamide solution by following the method of Yeung et al. and cast the hydrogels following steps described by Trepatt et al.^{14, 15}. One exception to the procedure is that the beads

embedded immediately underneath the cells are 0.5 μm in diameter and emit yellow fluorescence.

2. Skip the step of attaching 2 μm beads to the coverglass if the viewing area is expected to have a large cell-free region.

NOTE: Hydrogel preparation protocol is now fairly established in the field¹⁶. Throughout the subsequent description, the 0.5 μm beads are referred to as 'top beads', and the 2 μm beads are referred to as 'bottom beads'. However, the bottom beads are optional where the imaged region contains a large cell-free area. The top bead pattern from such a cell-free region will serve the purpose of the bottom bead pattern.

3. Mount the hydrogel with embedded fluorescent beads on the microscope stage.
4. Allow 15-20 min for the temperature of the plate to attain a steady-state.

NOTE: The plate's top surface is functionalized with extracellular matrix proteins, but the cells are not yet seeded on the hydrogel.

2. Reference image acquisition

1. Part 1: Creating a position list

NOTE: Manual input in this step includes following the AcTrM prompts to choose locations with the best bead distribution. No previously created files are used in this step. Key new folders produced in this step include 't0imgs', 'tnimgs', and 'textfiles' folders, and the file produced includes the position list file. See **Figure 2** for the visual description of the following steps that guide the creation of a position list using AcTrM. The demonstrated example acquires data at 20x magnification.

1. Start μ Manager 2.0 - Beta.
 2. Run AcTrM by clicking on the **AcTrM.bsh** button in the **IMBL Resources** window.
 3. In the window titled **Define Acquisition Properties**, select the appropriate objective, tolerance for the accuracy of lateral position (i.e., XY) recovery, step size of the rough and refined refocusing (i.e., z) operation, and value of acceptable image correlation coefficient for focus recovery.

NOTE: A finer tolerance for the lateral position recovery will slow position recovery, but a substantial tolerance will need position correction during data analysis and may cause difficulty in focus recovery. A smaller step size will slow the refocusing operation, but a very high step size may cause rapid movement with opportunities to miss the focus.
 4. In the **AcTrM - Step 0** window, select **Fresh Acquisition** to create a position list.

NOTE: The window titled **AcTrM - Step 1** lists all the key next steps. These steps involve moving the stage, adjusting the focus, and clicking on buttons in the μ Manager. These steps guide in constructing the list of positions suitable for data acquisition.
 5. Click on **Live** in **Micromanager** to visualize the samples for manual adjustments listed in the **AcTrM - Step 1** window.
 6. Follow the steps listed on this window.
 7. Now acquire the image of the beads. Select the appropriate channel for the top beads.
 8. Ensure to look at the bottom beads as well. To do so, select the channels for the bottom beads. A blurry image of bottom beads is seen.

NOTE: Channels can be switched from the preset dropdown menu on the main μ Manager window. If the bottom beads are used in the experiment, ensure that they are present in the selected position. These beads will appear blurry.
 9. Upon selecting the appropriate position, click on **Mark** in the **Stage Position List** window to save the position.

NOTE: The best position is defined by a dense and uniform distribution of the top beads embedded immediately underneath the top surface (i.e., top beads) and at least two beads attached to the cover glass (i.e., bottom beads) (**Supplementary Figure S1**). Focus recovery is faster if the bottom beads appear as large, out-of-focus rings. However, if experiments are done in one position with no need for focus or lateral position recovery, ignore instructions related to the bottom beads image.
 10. Follow steps 6-9 described in **Figure 2** to include additional positions into the list.

NOTE: Choose a few additional positions so that a round of elimination can allow for the best positions chosen on the plate.
2. **Part 2: Acquisition of reference images**

NOTE: This step does not involve any manual input. Previously created key files used in this step include `textfiles/*.pos`. Key new files and folders produced in this step include those within `t0imgs` and `tnimgs`

folders. See **Figure 3** and **Supplementary Figure S2** for the visual description of the following steps that guide the acquisition of reference images using AcTrM. The **AcTrM - Step 2** window also lists all the key steps.

1. Following the steps listed in **AcTrM - Step 2** window, make the choices in the **Multi-Dimensional Acquisition** window. For example, to perform a long-time lapse, indicate a number of images to be taken, select the first channel as phase channel, and then select the next for the top beads and the one after that is for bottom beads. Click on **Close** in the **Multi-Dimensional Acquisition** window, and then click on **OK** in the **AcTrM - Step 2** window.
2. In the **AcTrM - Step 3** window, choose to engage the reference-image-based XYZ position recovery. The choice is generally yes. However, if images are acquired at one position with no room for focus or stage drift, the choice in **AcTrM - Step 3** window will be **No**.
3. In the **AcTrM - XYZ Recovery Options** window, set the channel for rough XYZ recovery, whether to perform refined XYZ recovery, region, and channel for refined XYZ recovery and the direction to begin refocusing (**Supplementary Figure S4**). When completed, AcTrM will acquire reference images.

NOTE: Typical choices for the channel will be the bottom beads channel. XYZ recovery works best when carried out with the full image in the current implementation. Reference images will typically include a transmitted light image of the hydrogel, a fluorescent image of top

beads, and a fluorescent image of the bottom beads. Each image set's content may vary based on the choices made in the **Multi-Dimensional Acquisition** window. Still, the software will acquire the reference image set at each position determined in **Figure 2**. At the end of this step, three folders are created in the chosen directory: ``t0imgs'`, ``tnimgs'`, and ``textfiles'`. The ``t0imgs'` folder contains reference images in the format recognized by μ Manager and used in subsequent steps; ``tnimgs'` folder contains separate TIFF images with filenames ``c0_p0_t0.tif'`, ``c1_p0_t0.tif'`, etc. Here, ``c'` stands for the channel, ``p'` stands for the position, and ``t'` stands for the time. Following these letters are the channel number, position number, and frame number, respectively. The folder ``textfiles'` contains the position list in XML format and user choices for image acquisition and position recovery.

3. Cell seeding and growth

NOTE: The iTACS platform has the flexibility to accommodate sample preparation protocols used in common *in vitro* assessment of mechanical behavior of adherent cells, including sparse cells, fully confluent monolayer, network formation assay, and monolayers with holes or significant gaps.

1. Culture rat pulmonary microvascular endothelial cells to confluence in a flask^{17,18}.
2. Detach the cells using trypsin. Resuspend the cells in a culture medium containing 10% fetal bovine serum to a concentration 1×10^6 cells/mL.

3. Place a 5 μ L droplet of the resuspended cells on a partially dry hydrogel surface and place it in the cell culture incubator.

NOTE: After 2 days in the culture medium containing 10% fetal bovine serum, the cells in that droplet form a crowded island of cells¹.
4. **Automated image acquisition for the remaining experiment**

NOTE: Manual input for this step includes following the AcTrM prompts to resume the image acquisition. Key previously created files used by this step include `textfiles/*.pos` and bottom bead image files in the folder `t0imgs`. Key new files produced in this step include updated position list files and images `tnimgs/*_t*.tif`.

 1. Ensure that the microscope environmental control system reaches stable tissue culture conditions.
 2. Gently mount the plate containing cultured cells onto the microscope stage.
 3. Allow 15-20 min for the temperature and humidity to stabilize.

NOTE: See **Figure 4** for the visual description of the following steps that guide the acquisition of images for the remaining experiment using AcTrM.
 4. Start **μ Manager 2.0 - Beta**.
 5. Run AcTrM by clicking on the **AcTrM.bsh** button in the **IMBL Resources** window.

NOTE: Ignore the choices made in the **Define Acquisition Properties** window but use the choices made in the previous step.
 6. In the **AcTrM - Step 0** window, select **Continue Paused Acquisition**.
 7. Select the magnification and directory identified in the **Multi-Dimensional Acquisition** window in the earlier step where data folders (`t0imgs`, `tnimgs`, and `textfiles`) were saved.

NOTE: The title of the window guides the user to choose the appropriate directory.
 8. In the **Repositioning the Plate** window, select choices for repositioning the plate (three noncollinear positions) along with the channel used for repositioning.

NOTE: Saved images will be seen in the camera. If overlapping images display as a red, green, and black color image, perform manual adjustment.
 9. Click on **Accept** to proceed with the acquisition.

NOTE: This step overcomes the slight shifts from the irreproducibility of plate alignment when the plate is remounted on the microscope stage. Follow AcTrM prompts to expedite alignment at each of the three positions by showing a composite image of the reference image shown in red (typically of the bottom beads) and the image currently observed through the objective shown in green. Getting the green sufficiently close to red leaves less work for the software. When the overlap is perfect, the composite image appears yellow and black. Sufficiently close is typically when the same bottom beads are visible in both red and green images, and the corresponding red and green out-of-focus rings are touching each other. After the plate repositioning is complete, AcTrM takes the stage to each selected position and recovers XYZ position by matching the reference image with what is currently seen through the camera. The first lateral (XY) position is matched, and then the focus (Z) is matched. Rough

recovery is followed by refined recovery of the position and focus. Updates of the current status of the experiment are displayed on the screen through a four-part window shown in **Supplementary Figure S3**. The acquired images are saved in the `tnimgs` folder following `*_t1.tif`, `*_t2.tif`, indicating the time count for the image set. If XYZ recovery identifies an updated position, the new position list is generated and saved in the `textfiles` folder.

2. Analysis and Visualization Module (AnViM)

1. Setting up automated data analysis

NOTE: Manual input in this step includes identifying the location of the `tnimgs` folder. Key previously created files used in this step include `tnimgs/*.tif`. Key new files and folders produced in this step include the `analysis` folder in the same parent folder as `tnimgs` and folders for each position `analysis/p*`. Within each `analysis/p*`, this step creates a new `*.tif` image files within `phs` and `tny` folders. These images are cropped and de-drifted images of cells and top beads. The other files created include `analysis/analysisChoices.txt` which lists analysis choices, `analysis/p*/skipAnalysis.txt`, which lists instances where analysis is skipped due to significant preexisting drift, and `analysis/p*/part_shift_values_pixel_degree.dat` which lists estimated drift values. AnViM does not alter the raw data in the `tnimgs` folder. See **Figure 5** for the visual description of the following steps that guide the setting up of automated data analysis using AnViM.

1. Start the Fiji software and select the first option in the **MSM** drop-down menu labeled as **MSM - pre-processing**.

2. Using the file browser dialog, select the folder containing the `tnimgs` folder, which contains the analyzed data.
3. Define the channel for the images. Following the guidelines from **Supplementary Figure S5**, identify the channel numbers of the transmitted light image of the cells (phase contrast image of the cells), bottom beads image and top beads image. The following three checkboxes ('Move files to the position folder', 'Do additional correction for rigid motion', and 'Crop data and save in position folder') are typically checked. Finally, define tolerance for rejecting heavily drifted data, pixel size, and sides of the image where the cells cross.
4. Respond to the prompt for enhancing the brightness and contrast of the bottom bead images so the beads appear prominently. Adjust this using the slider bar in the menu and click on **OK**.
5. Now perform position correction to get rid of the shifts, if any. Once done, an analysis folder is created.

NOTE: Contrast enhancement is typically not needed, but this provision is available for experiments where exposure to lasers needs to be minimized. After that choice, AnViM copies the files from the `tnimgs` folder to the `analysis` folder. The files corresponding to each position are saved in folders `analysis/p0`, `analysis/p1`, etc. Within each of these position folders, AnViM makes `cels`, `defs`, and `refs` folders containing the original transmitted light image, top beads images, and bottom beads images, respectively (**Supplementary Figure S6**). AnViM then analyzes rigid motion in the images

of the bottom beads and creates a `phs` folder containing a corrected transmitted light image of the cells and a `tny` folder containing updated top beads images. Finally, user choices of the channels, operations, tolerance, pixel size, and boundary-crossing are saved in the `analysisChoices.txt` file (**Supplementary Figure S6**).

2. Quantification of deformation of hydrogel and monolayer

NOTE: It is important to note that quantification of movement from a sequence of images is a rapidly evolving field¹⁹. The technology is constantly optimized for features, including speed, accuracy, specific features within the raw images, and specific deformation patterns. Hence, it is likely that some users may use a different deformation quantification approach than the one presented here.

1. Part 1: Quantification of hydrogel deformation

NOTE: Manual inputs in this step include identifying data directory and selection of grid resolution. Key previously created files used in this step include `p*/tny/*.tif`. Key new files produced in this include `p*/displacement/*_disp.dat` that lists displacement vectors of the top surface of the hydrogel. See **Figure 6** for the visual description of the following steps to engage, *via* AnViM, the Particle Image Velocimetry analysis on the top beads images²⁰.

1. Start the Fiji software, and from the **MSM** drop-down menu, select **MSM - Gel Deformation**.
2. From here, select the option that is suitable for the experiment.

3. Using the file browser dialog, select the parent directory of the `analysis` folder containing the analyzed data.
4. In the **Parameters for Calculating Gel Deformation** window, select appropriate cross-correlation window size, noise level, and threshold (**Supplementary Figure S7**)²⁰.

NOTE: The results are saved in a newly created `displacement` directory within each position folder `analysis/p0`, `analysis/p1`, etc. This is where all the output files are stored.

2. Part 2: Quantification of monolayer deformation

NOTE: Manual inputs in this step include identifying the data directory and selection of grid resolution. Key previously created files used in this step include `p*/tny/*.tif`. Key new files produced in this include `p*/velocity/*_vel.dat` that lists motion vectors of the cells. See **Figure 7** for the visual description of the steps to engage, *via* AnViM, the Particle Image Velocimetry analysis on the top beads images²⁰. The procedure is similar to the one followed for quantifying hydrogel deformation and selects **MSM - Cellular Motion** from the **MSM** drop-down menu. The results are saved in a newly created `velocity` directory within each position folder `analysis/p0`, `analysis/p1`, etc.

3. Quantification of cell-ECM and cell-cell forces

NOTE: Manual input in this step includes identifying the data directory, indicating the hydrogel stiffness, and responding to cellular cluster segmentation prompts to detect cells from the no-cell region. Key previously created files used in this step include `p*/displacement/*_disp.dat`. Key new files produced in this step include:

`p*/traction/*_trac.dat', which lists forces exerted by the cells on the hydrogel; `p*/traction/*_domain.dat', which lists the location of grid points containing cells; and input files `p*/traction.in, p*/clusterInput.txt' and `p*/prestress.in' that record user choices for this step. Following the first quantitative assessment of cell-ECM and cell-cell forces, several variations to the technique have been developed^{1,15}. The variations focus on particular cases of substrates, cells, experimental conditions, or numerical tools^{7,8,21,22}. See **Figure 8** for the visual description to engage *via* AnViM, the Fourier Transform Traction Microscopy, and Monolayer Stress Microscopy analysis on the hydrogel deformation data^{1,2,15}.

1. Start the Fiji software, and from the **MSM** dropdown menu, select **MSM - Cell-ECM and Cell-Cell Forces** (third option in the dropdown menu).
2. Using the file browser dialog, select the parent directory of the `analysis' folder containing the folders `tnimgs' and `analysis' containing analyzed data. Click on **Select**.
3. In the **Parameters for Calculating Cellular Forces** window, enter the shear modulus, thickness of the hydrogel and the anticipated noise level. Select **OK**. This lets it execute traction *via* the MATLAB function. **NOTE:** Following these inputs, AnViM computes cell-ECM forces.
4. After that, indicate whether the monolayer is confluent. If the entire cell image is covered with cells, then the answer is **Yes**. In that case, the cell-cell forces will be computed across the whole frame. On the other hand, if a portion of the image does not have cells, then the answer is **No**. In that case,

follow AnViM prompts to facilitate segmentation of the image region that contains cells.

5. If the answer is no, manually draw a polygon around the non-cell object when the software prompts to do so, and then select an appropriate method(s) for segmentation. The software will ask for the color (black or white) of the cells.
6. Check the **Fill Spots Automatically** option in the software and click on **OK**.

NOTE: The steps for segmenting a non-confluent monolayer will be covered in the first part of `Section 2.4. Cell-ECM forces are stored in `*_trac.dat ' files in a newly created directory `traction' within each position folder, and the input to the cell-ECM force calculation software is saved in the file `traction.in' (**Supplementary Figure S8**). Cell-cell forces are stored in `*_prestress.dat' files in a newly created directory `prestress' within each position folder, and the input to the cell-cell force calculation software is saved in the file `prestress.in' (**Supplementary Figure S9**).

4. Mapping grid point values on individual cells

NOTE: One of the current focuses of iTACS is to introduce a straightforward approach to interpret the measured mechanical signals in the field. This approach is beneficial for examining mechanical interactions among neighboring cells of a cluster¹⁸. Overall, the properties quantified until now are on regularly spaced grid points across the cellular cluster. This data identifies median, mean, and standard deviation of selected properties within the morphological boundary of individual cells and assigns those as cellular physical properties/signals. From these, the standard deviation is used to indicate variability, the difference between mean

and median is used to indicate the nature of distribution, and the median value is used to indicate the overall state of the cells for the selected properties.

1. Part 1: Segmentation of the image region that contains cells

NOTE: Manual input to this step includes identifying data directory and responding to the segmentation prompts to detect cells from the no-cell region. Key previously created files used in this step include `p*/phs/*.tif`. Key new files produced in this step include `p*/phs/bwImages/*.tif` which are binary images separating cell regions from no-cell regions, `p*/phs/textResults/frameNum_percentHealed.txt`, which lists percent image area covered by cells in each instance, and `p*/clusterInput.txt`, which records user choices entered in the AcTrM interface. See **Figure 9** for the visual description of the following steps to segment the image region that contains cells. This part must be completed before computing the cell-cell forces. In that case, these steps need not be repeated. Also, if these steps are conducted before the cell-cell force calculations, they are not requested at the beginning of the force calculations.

1. Start the Fiji software, and from the **MSM** drop-down menu, select **Segmentation - Cellular Cluster**.
2. Using the file browser dialog, select the position directory `analysis/p0`, `analysis/p1`, etc., that contains properties quantified on regularly spaced grid points.
3. Indicate whether the monolayer is confluent.

NOTE: The steps below are invoked only when the monolayer is not confluent. Follow AnViM prompts to apply the novel multi-pronged approach to identify the image regions that contain cells. The process involves four methods - each approaching segmentation in a different manner. One or multiple of these methods used in combination cover a wide variety of images of the cells. Therefore, try different approaches to find which combination works best for their data.

4. Adjust the `Domain connection factor` and `Smear factor` to obtain optimum segmentation. **NOTE:** The `Smear factor` makes the regions that contain cells larger.

5. Indicate whether the cells appear black or white in the binary image.

6. In the **Directions to Clean the Boundary Image** window, choose whether to clean the image automatically or manually.

NOTE: Unwanted features of the images are black spots on the pixels occupied by cells and white spots on the pixels disconnected from the cell cluster to be analyzed. Analysis can only be done on the regions that are connected. So multiple disconnected regions must be analyzed separately.

2. Part 2: Segmentation of the individual cells in the images

NOTE: Manual inputs to this step include identifying the data directory and responding to the segmentation prompts to detect individual cells within the image. Key previously created files

used in this step include `p*/phs/*.tif` and `p*/phs/bwImages/*.tif`. Key new files produced include `p*/phs/textResults/*.csv`, which contain cellular morphological properties. See **Figure 10** for the visual description of the following steps to segment individual cells of the monolayer.

1. Start the Fiji software, and from the **MSM** drop-down menu, select **Segmentation - Individual Cells**.
2. Using the file browser dialog, select the position directory `analysis/p0`, `analysis/p1`, etc., that contains properties quantified on regularly spaced grid points.
3. In the **Choose the Input Parameters** window, indicate the maximum aspect ratio, prominence of how much the cell center stands out compared to the cell boundary, and two blurring parameters to guide the detection of the cells (**Supplementary Figure S10**).
4. On the image stack for each position, draw a polygon on the smallest normal cell. This is used to calculate the area. Anything smaller than this will not be considered as a cell by the software.
5. Next draw a polygon around the largest normal cell and indicate whether the cell center or the cell-cell interface is brighter.

NOTE: AnViM then creates files `phs/textResults/0001.csv`, `phs/textResults/0002.csv`, etc., for each frame containing information on cells within that frame. At this stage, this file includes morphological information about the cells, including area, centroid, perimeter, orientation, circularity,

aspect ratio, roundedness, solidity, distance from the no-cell region. The length unit in these properties is pixels, and the angle is in degrees. Finally, this file is updated to contain cellular pixel intensity, motion, and forces in the subsequent steps.

3. Part 3: Mapping of the pixel intensities on the cells

NOTE: Manual inputs in this step include identifying the data directory and selecting mapping properties and parameters. Key previously created files used in this step include `p*/phs/*.tif` (or `p*/fluo/*.tif`) and `p*/phs/bwImages/*.tif`. Key new files produced in this step include `p*/phs/textResults/*.csv`, which lists cellular morphological properties. See **Figure 11** for the visual description of the following steps to assess the pixel intensities in the region encompassed by individual cells and their neighboring region and define them as properties of the cells.

1. Start the Fiji software, and from the **MSM** drop-down menu, select **Map on Cells - Intensities**.
2. Using the file browser dialog, select the position directory `analysis/p0`, `analysis/p1`, etc., that contains properties quantified on regularly spaced grid points.
3. Select Detect Cell Division or Quantify Cellular Fluorescence when prompted by the software. Define the size of the neighboring region. Check both Collect Properties of the Neighboring Region and Collect Properties of the Cells boxes. Click on **OK**.

- In the Purpose of Recording the Cellular Intensity window, indicate what type of image to be used for intensity mapping, the size of the neighboring region, and whether data is collected for individual cells or both cells and their neighboring regions (**Supplementary Figure S11**).

NOTE: Mapping the pixel intensities of a phase-contrast image allows detection of cell division events. Mapping fluorescent image intensities will enable detection of fluctuations in the fluorescently tagged cytoplasmic molecules. The output of the above steps is the mean, median, standard deviation, minimum, and maximum pixel intensity values for individual cells. These numbers are entered as new columns in the files `phs/textResults/0001.csv`, `phs/textResults/0002.csv`, etc.

4. Part 4: Mapping of the forces and motion on the cells

NOTE: Manual inputs to this step include identifying the data directory and selecting mapping properties and parameters. Key previously created files used in this step include `p*/phs/textResults/*.csv`, `p*/velocity/*_vel.dat`, `p*/displacement/*_disp.dat`, `p*/traction/*_trac.dat`, `p*/prestress/*_prestress.dat`, and `p*/phs/bwImages/*.tif`. No new files are created during this step. Instead, the new information (cellular force and motion properties) are added to the files `p*/phs/textResults/*.csv`. See **Figure 12** for the visual description of the steps to assess the forces and motion in the region encompassed by individual cells and their

neighboring region and define them as properties of the cells.

- Choose from the **MSM** drop-down menu **Map on Cells - Motion/Force Data (Supplementary Figure S12)**.
- Then, follow the steps listed in step 2.4.3.
NOTE: New columns are added to the files `phs/textResults/0001.csv`, `phs/textResults/0002.csv`, etc. and include the mean, median, and standard deviation of speed, the orientation of velocity, average cytoskeletal tension, tension anisotropy, strain energy in the hydrogel, the orientation of highest tension, and magnitude of cell-ECM traction (**Supplementary Figure S13**).

5. Visualization of results

1. Part 1: Tracking of the cellular identities across the experiment

NOTE: Manual inputs to this step include identifying the data directory and selecting mapped properties to visualize. Key previously created files used in this step include `p*/phs/textResults/*.csv`. Key new files produced during this step include `p*/phs/textResults/cellTimeTrace_*.csv`, which contains a time trace of the cellular data. See **Figure 13** for the visual description of the following steps to track the properties of individual cells over the entire duration of the experiment.

- Start the Fiji software, and from the **MSM** drop-down menu, select **Results - Track Data**.
- Using the file browser dialog, select the position directory `analysis/p0`, `analysis/p1`,

etc., containing cellular properties to be tracked.

When done click on **Select**.

3. In the **Choose Starting Point for Tracking** window, choose the starting frame for monitoring, and the number of frames simultaneously tracked (**Supplementary Figure S14**). Always start from frame number 2 as velocity cannot be determined for frame number 1. When done, click on **OK**.

4. In the **Check the Variables to Make Tracks** window, choose the variables either by typing the variable names or selecting the terms from the checkboxes (**Supplementary Figure S15**). Then, click on **OK**.

NOTE: Tracking generates `phs/textResults/cellTimeTrace_*.csv` files with each column containing a unique cell number and each successive row representing the consecutive time instance.

2. Part 2: Generation of time tracks of the assessed cellular properties

NOTE: Manual inputs to this step include identifying the data directory and selecting mapped properties to visualize. Key previously created files used in this step include `p*/phs/textResults/cellTimeTrace_*.csv`. Key new files produced during this step include `p*/phs/trackedDataPlots/*.tif`, which contain time track plots and `p*/phs/trackedDataPlots/*.csv`, which contain plot data. See **Figure 14** for the visual description of the following steps to generate time tracks of the assessed cellular properties.

1. Start the Fiji software, and from the **MSM** drop-down menu, select **Results - plot**.
2. Using the file browser dialog, select the position directory `analysis/p0`, `analysis/p1`, etc., containing tracked cellular properties to be plotted.
3. Choose whether to limit the plotting to cells with unbroken tracks by clicking on the **Yes** or **No** button.

NOTE: This option ignores the cells, which could not be detected in one or more time instances.

4. Choose the number of variables to be plotted simultaneously and click on **OK**.

NOTE: Currently, AnViM allows a maximum of three variables to be displayed in the same plot.

5. Choose individual variables for plotting using a drop-down menu.

NOTE: These steps create a tagged image file format (TIFF) file and a comma-separated data file within the `phs/trackedDataPlots/` folder. The filename is composed of variable names separated by an underscore and ends with a cell number.

3. Part 3: Generation of heat maps of the assessed cellular properties

NOTE: Manual inputs in this step include identifying the data directory and selecting mapped properties to visualize. Key previously created files used in this step include `p*/phs/textResults/cellTimeTrace_*.csv`. Key new files generated in this step include `p*/phs/segmentedImages/cellPropMaps/*.tif`, which are the heatmaps of

cellular properties. See **Figure 15** for the visual description of the steps to generate heat maps of the assessed cellular properties.

1. Choose from the **MSM** drop-down menu **Results - Picture**.
2. Follow the steps described in step 2.5.2.

NOTE: The output is stored as TIFF file files in the `phs/segmentedImages/cellPropMaps/` folder. The filenames are the time instance number and variable name separated by an underscore.

Representative Results

We present here two of the key outputs for the demonstrated example. The first output is the time trace of cellular speed and cytoskeletal tension for cell number 1 (**Figure 16**). The properties are shown on a shared vertical axis to facilitate the visual association between the properties, and the horizontal axis indicates time instance number. In this experiment, successive frames were acquired at a 15 min interval. The second output is an array of heat maps 1 h into the experiment (**Figure 17**). The properties shown here include spread area, orientation, circularity, speed, direction of motion, maximum tension orientation, cytoskeletal tension, substrate tractions, and tension anisotropy of individual cells.

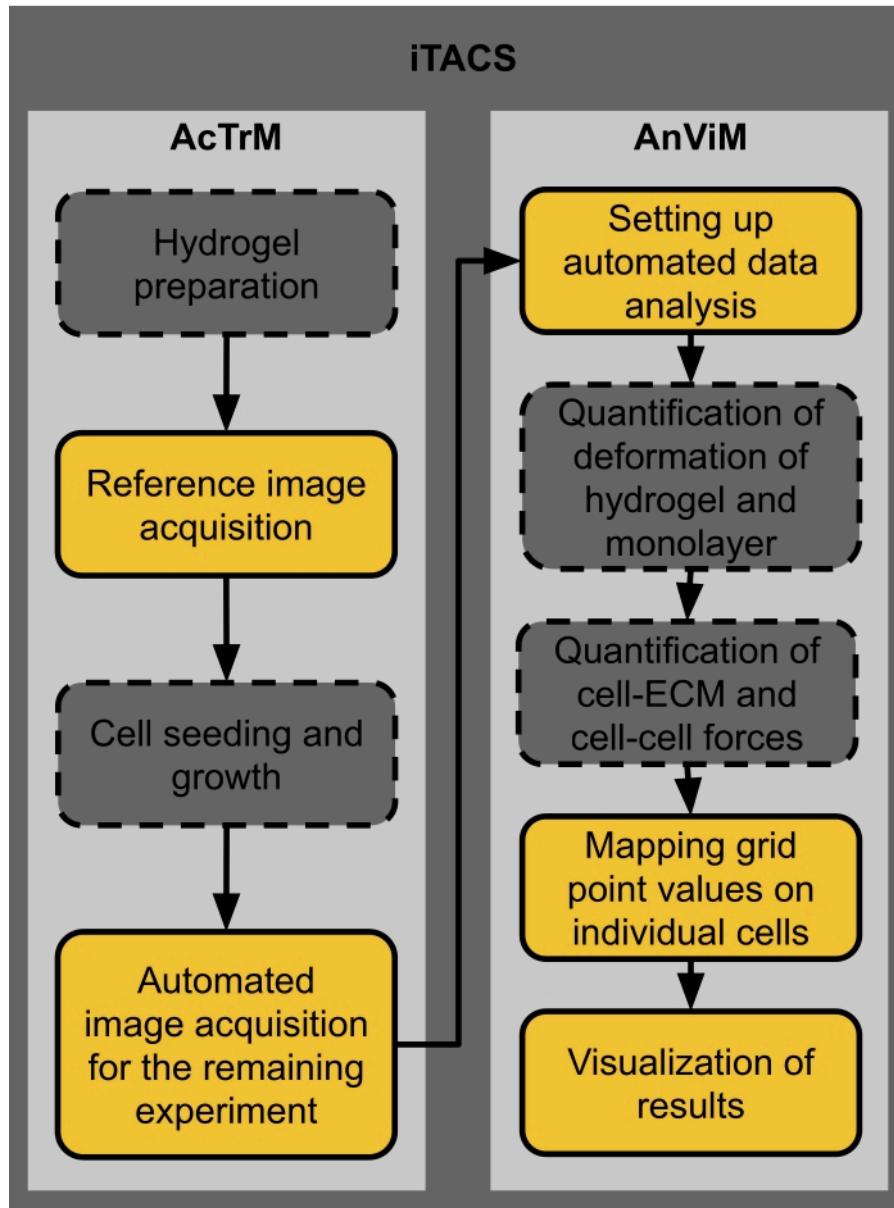


Figure 1: Structure of Integrative Toolkit to Analyze Cellular Signals (iTACS). Two key components of iTACS are Acquisition and Training Module (AcTrM) and Analysis and Visualization Module (AnViM). AcTrM can use various hydrogel preparation techniques that currently exist for preparing hydrogels that can be held firmly on a microscope stage, any cell seeding, and a growth protocol that retains cells in one focal plane. AnViM can use various techniques to quantify the hydrogel and monolayer deformation, cell-ECM forces, and cell-cell forces. All these user-preferred components of the force measurements protocol can be accommodated in iTACS, and they have been identified with dashed boxes. The components identified with solid boxes are novel contributions to cellular force measurement technology. Visualization in the AnViM

focuses on the median value and variability of the properties across individual cells. [Please click here to view a larger version of this figure.](#)

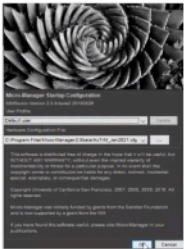
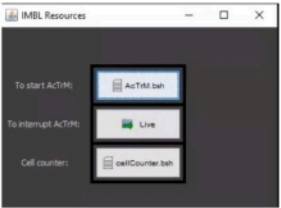
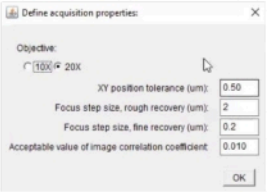

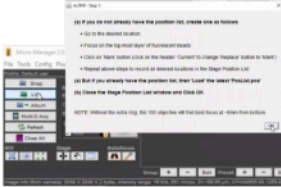
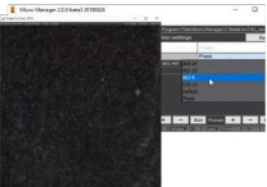
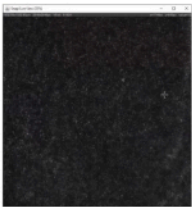
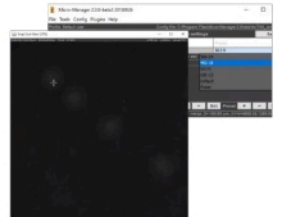
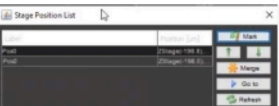
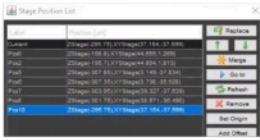
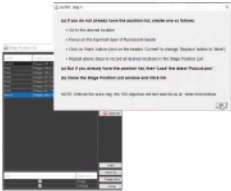
<p>1 Open MicroManager.</p> 	<p>2 Start AcTrM by clicking AcTrM.bsh.</p> 	<p>3 Select appropriate objective and other hardware parameters for the experimental set up. Click OK.</p> 
<p>4 AcTrM - Step 0 prompts user to select the acquisition type: fresh (starting an experiment), continued (resuming earlier experiment).</p> 	<p>5 Click Live in Micro-Manager to visualize the sample for manual adjustments. AcTrM - Step 1 lists the next steps.</p> 	<p>6 Select channel appropriate for the top beads (563-9). Go to a desired position. Ensure that the bead distribution is homogeneous and dense.</p> 
<p>7 Move the focal plane above the top beads and slowly lower until the first instance of beads appear in focus.</p> 	<p>8 Select channel appropriate for the top beads (492-18). Ensure that the position contains at least two beads.</p> 	<p>9 In Stage Position List, click Mark.</p> 
<p>10 Repeat steps 6-9 for each well. Recorded stage positions will be listed in the Stage Position List.*</p>  <p>*To review selected stage positions, select a position listed, then select Go in the side menu to view position in SnapiLive View. *To remove a stage position, select Remove in the side menu.</p>	<p>11 Measurements will be taken at these locations. Click Close in the Stage Position List side menu, and click OK in AcTrM - Step 1.</p> 	

Figure 2: Reference image acquisition - part 1. Steps for creating a position list using AcTrM. [Please click here to view a larger version of this figure.](#)

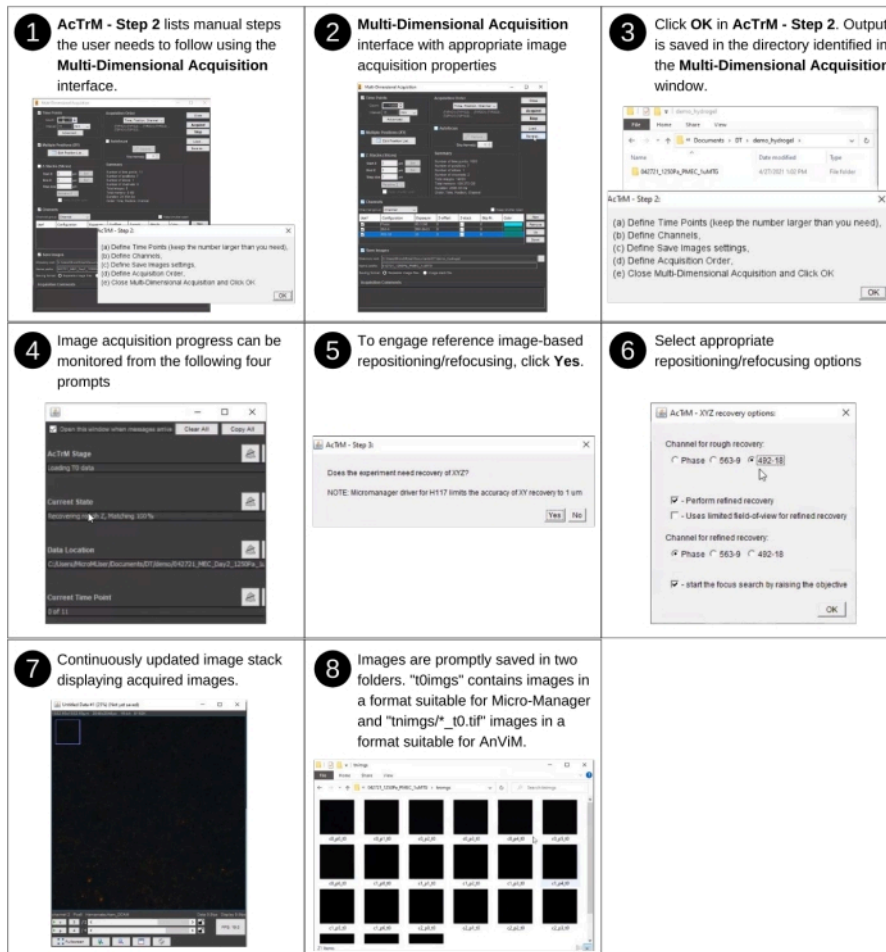


Figure 3: Reference image acquisition - part 2. Steps for acquiring reference images using AcTrM. Detailed views of steps 2, 4, and 6 are presented in **Supplementary Figures S2, S3, and S4**, respectively. [Please click here to view a larger version of this figure.](#)

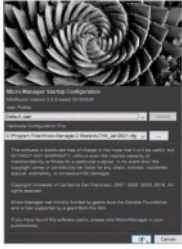
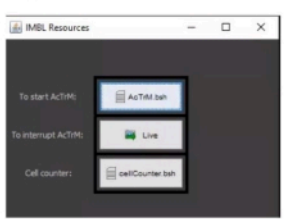
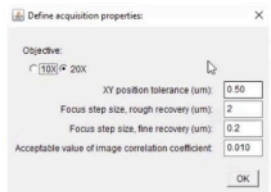
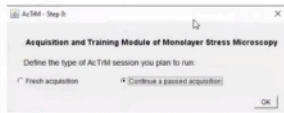
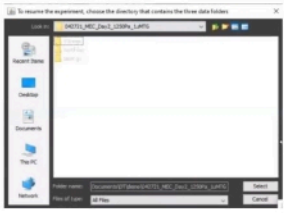
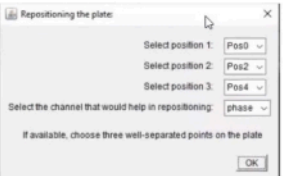

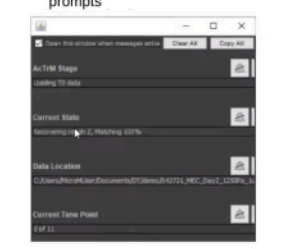
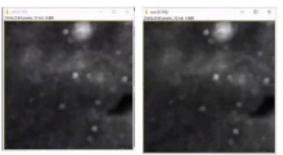

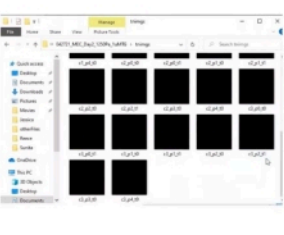
<p>1 Open MicroManager.</p> 	<p>2 Start AcTrM by clicking AcTrM.bsh.</p> 	<p>3 Select appropriate objective and other hardware parameters for the experimental set up. Click OK.</p> 
<p>4 Select Continue a paused acquisition and click OK.</p> 	<p>5 Select the directory that contains three folders (0Images, textFiles, and tnings) and click Select.</p> 	<p>6 Select three non-colinear positions from the automatically loaded position list. Select the channel used to guide repositioning of the plate and click OK.</p> 
<p>7 Adjust large shifts using MicroManager's Stage Control window. Click Refresh to view alignment after manual shifts. Click Accept when the image is mostly black and yellow.</p> 	<p>8 Plate repositioning progress can be monitored from the following four prompts</p> 	<p>9 Plate repositioning progress will be displayed with the reference image (left) and the current image (right). When satisfied, click OK! will seek response two more times.</p> 
<p>10 After plate repositioning, images will be acquired following repositioning/refocusing operations at each position.</p> 	<p>11 Images (c*_p*_t*.tif) saved in the "tnings" folder.</p> 	

Figure 4: Automated image acquisition for the remaining experiment. Steps for resuming image acquisition to assess cellular behavior using AcTrM. [Please click here to view a larger version of this figure.](#)

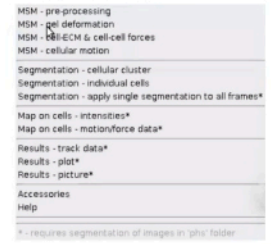
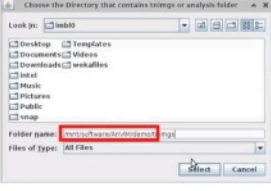
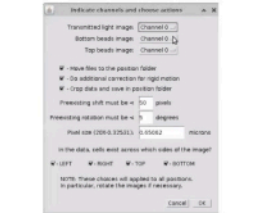
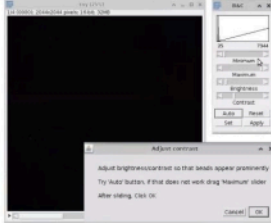
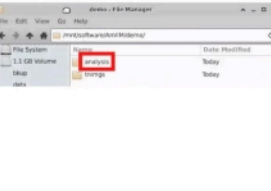

<p>1 From the MSM menu, click MSM - pre-processing.</p>  <p>MSM - pre-processing MSM - gel deformation MSM - Hi-CM & cell-cell forces MSM - cellular motion</p> <p>Segmentation - cellular cluster Segmentation - individual cells Segmentation - apply single segmentation to all frames*</p> <p>Map on cells - intensities* Map on cells - motion/force data*</p> <p>Results - track data* Results - plot* Results - picture*</p> <p>Accessories Help</p> <p>* - requires segmentation of images in 'p0' folder</p>	<p>2 Select the folder that contains the AcTrM-generated "tnimgs" directory.</p>  <p>Choose the Directory that contains tnimgs or analysis folder</p> <p>Look in: /mnt/b0</p> <p>desktop Templates Documents Videos Downloads Downloads Intel Music Pictures Public tmp</p> <p>Folder name: /mnt/b0/p0/tnimgs/analysis.tif</p> <p>Files of type: All Files</p> <p>Select Cancel</p>	<p>3 Select appropriate channel numbers and other experimental parameters. Click OK.</p>  <p>Define channels and choose options</p> <p>Top bead image: Channel 0 Bottom bead image: Channel 0 Top beads image: Channel 0</p> <p>Move files to the parent folder Do additional correction for registration Crop data and save in parent folder</p> <p>Preprocessing shift must be: 0 degrees Preprocessing rotation must be: 0 degrees Pixel size (X&Y): 0.556x0.250 microns</p> <p>In the data, cells exist across which sides of the image? LEFT RIGHT TOP BOTTOM</p> <p>NOTE: These choices will apply to all positions. In particular, rotate the images if necessary.</p> <p>Cancel OK</p>
<p>4 If needed, use sliders from the B&C window to enhance contrast of the bottom bead images, then click OK.</p>  <p>Adjust brightness/contrast so that beads appear prominently. To Auto button, if that does not work drag 'Maximum' slider. After clicking, click OK.</p>	<p>5 The pre-processing function will create an "analysis" folder with appropriate contents</p>  <p>File Manager</p> <p>File System 1.5 GB Volume tmp data</p> <p>analysis tnimgs</p> <p>Date Modified Today Today</p>	<p>6 Pre-processing completion prompt. Click OK.</p>  <p>Pre-processing done Make a backup</p> <p>Check the alignment of the files in 'p0' for later. Also, check the 'p0' folder for any remaining files. Use enhancement (L, R) channel and saving. You can also use 'analysis' mode and save data preparation.</p> <p>OK</p>
<p>7 Review top bead images (analysis/p0/tny/*.tif) to ensure that the images are de-drifted. To review files:</p> <ol style="list-style-type: none"> 1. Select an image file path. 2. Select File on the ImageJ option bar. Import Image Sequence will appear. 3. Click OK. 		

Figure 5: Setting up automated data analysis. Steps to begin automated image analysis using AnViM. The software recognizes the image format used by AcTrM. A detailed view of the panels in steps 3 and 5 is presented in **Supplementary Figure S5** and **Supplementary Figure S6**, respectively. [Please click here to view a larger version of this figure.](#)

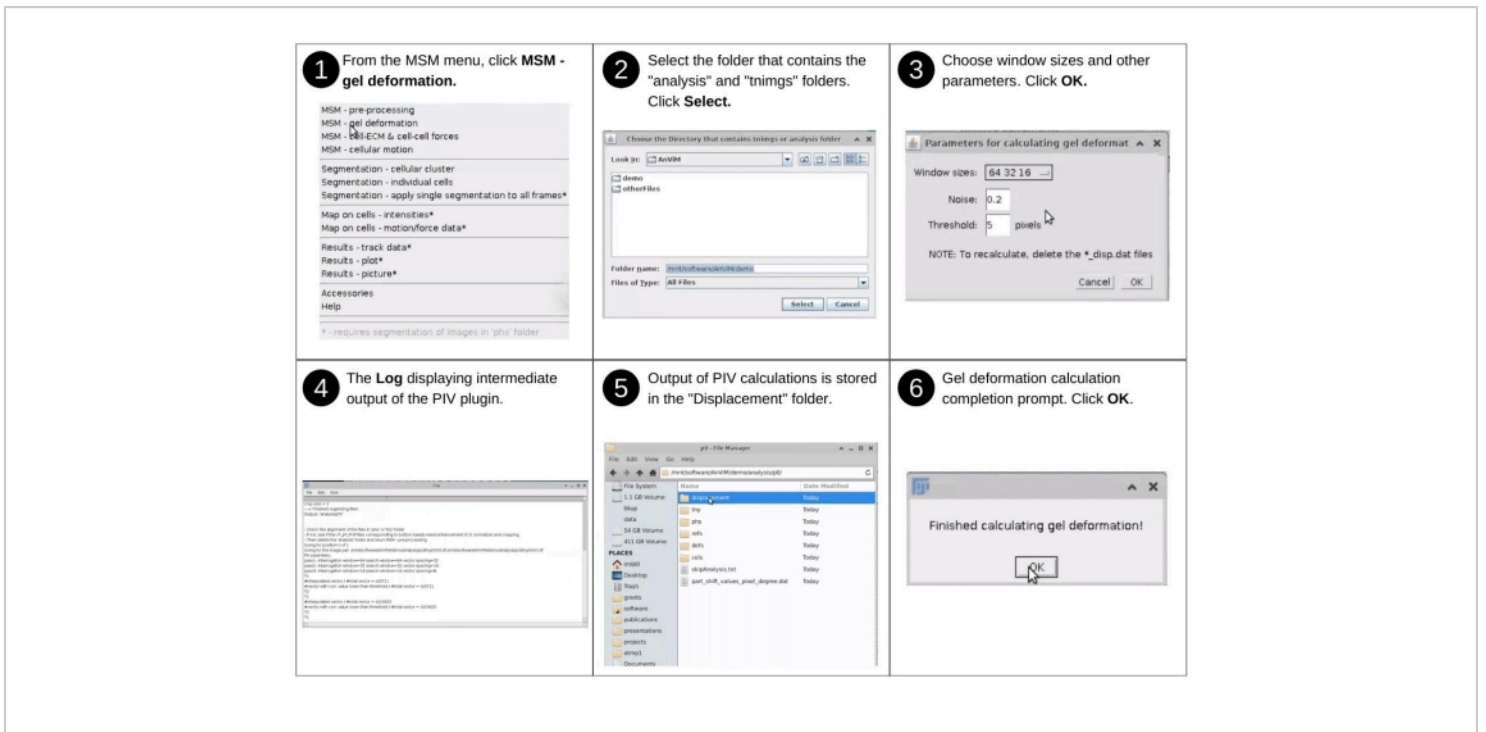


Figure 6: Quantification of deformation of hydrogel and monolayer - part 1. Steps to engage, via AnViM, the Particle Image Velocimetry implementation of Tseng, Q. et al., PNAS (2012)²⁰ to quantify deformation of the top surface of the hydrogel. Users can also implement within AnViM other approaches to quantify hydrogel deformation. A detailed view of step 3 is presented in **Supplementary Figure S7**. [Please click here to view a larger version of this figure.](#)

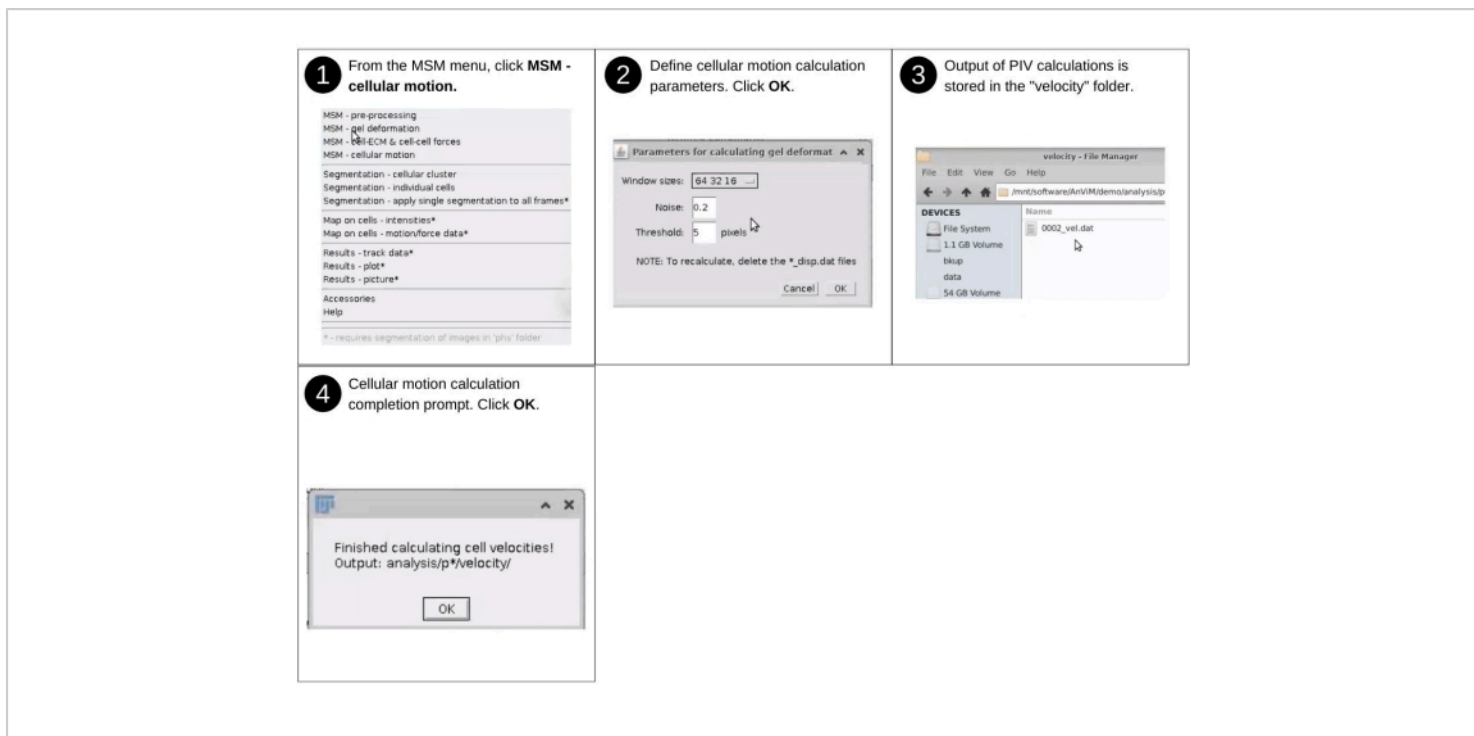


Figure 7: Quantification of deformation of hydrogel and monolayer - part 2. Steps to engage, via AnViM, the Particle Image Velocimetry implementation of Tseng, Q. et al., PNAS (2012)²⁰ to quantify the local motion of individual cells. Users can also implement within AnViM other approaches to quantify cellular motion. [Please click here to view a larger version of this figure.](#)

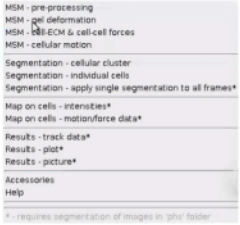
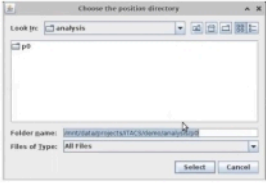
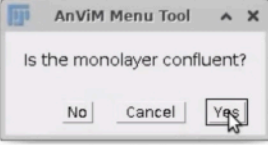

<p>1 From the MSM menu, click Segmentation - cellular cluster.</p>  <p>MSM - pre-processing MSM - gel deformation MSM - BE-ECM & cell-cell forces MSM - cellular motion Segmentation - cellular cluster Segmentation - individual cells Segmentation - apply single segmentation to all frames* Map on cells - intensities* Map on cells - motion/force data* Results - track data* Results - plot* Results - picture* Accessories Help * requires segmentation of images in 'ph' folder</p>	<p>2 Select the folder that contains the "position directory" or "p0". Click Select.</p> 	<p>3 Indicate if the cell monolayer is confluent. If No, move on to step 4; if Yes, move on to step 6.</p> 
<p>4 Indicate variation between one region and another and the blur factor. Select desired methods to separate cells from no-cell region. When finished, click OK.</p> 	<p>5 Indicate the desired color of cells for each method.</p> 	<p>6 Follow instructions to clean binary image. When finished, click OK.</p> 
<p>7 Output is stored in an image format (phs/bwImages/*.tif) And in a text format (phs/textResults/frameNum_percentHealed.txt).</p> 		

Figure 9: Mapping grid point values on individual cells - part 1. Steps to segment the image regions containing cells using a novel multi-pronged approach. This approach can be used to segment phase contrast, brightfield, or fluorescence images of the cells. [Please click here to view a larger version of this figure.](#)

1 From the MSM menu, click **Segmentation - individual cells**.

2 Define segmentation calculation parameters. Click **OK**.

3 Draw a polygon around the smallest normal cell. Click **OK**.

4 Draw a polygon around the largest normal cell. Click **OK**.

5 Indicate whether the cell-cell interface (boundary around the cell) or the cell center is brighter. Click **OK**.

6 Output of segmentation calculations is stored in the "textResults" folder.

7 Segmentation calculation completion prompt. Click **OK**.

Figure 10: Mapping grid point values on individual cells - part 2. Steps to segment individual cells of a monolayer using a novel multi-pronged approach developed in AnViM. This approach can be used to segment phase contrast, brightfield, or fluorescence images of the cells. A detailed view of step 2 is presented in **Supplementary Figure S10**. [Please click here to view a larger version of this figure.](#)

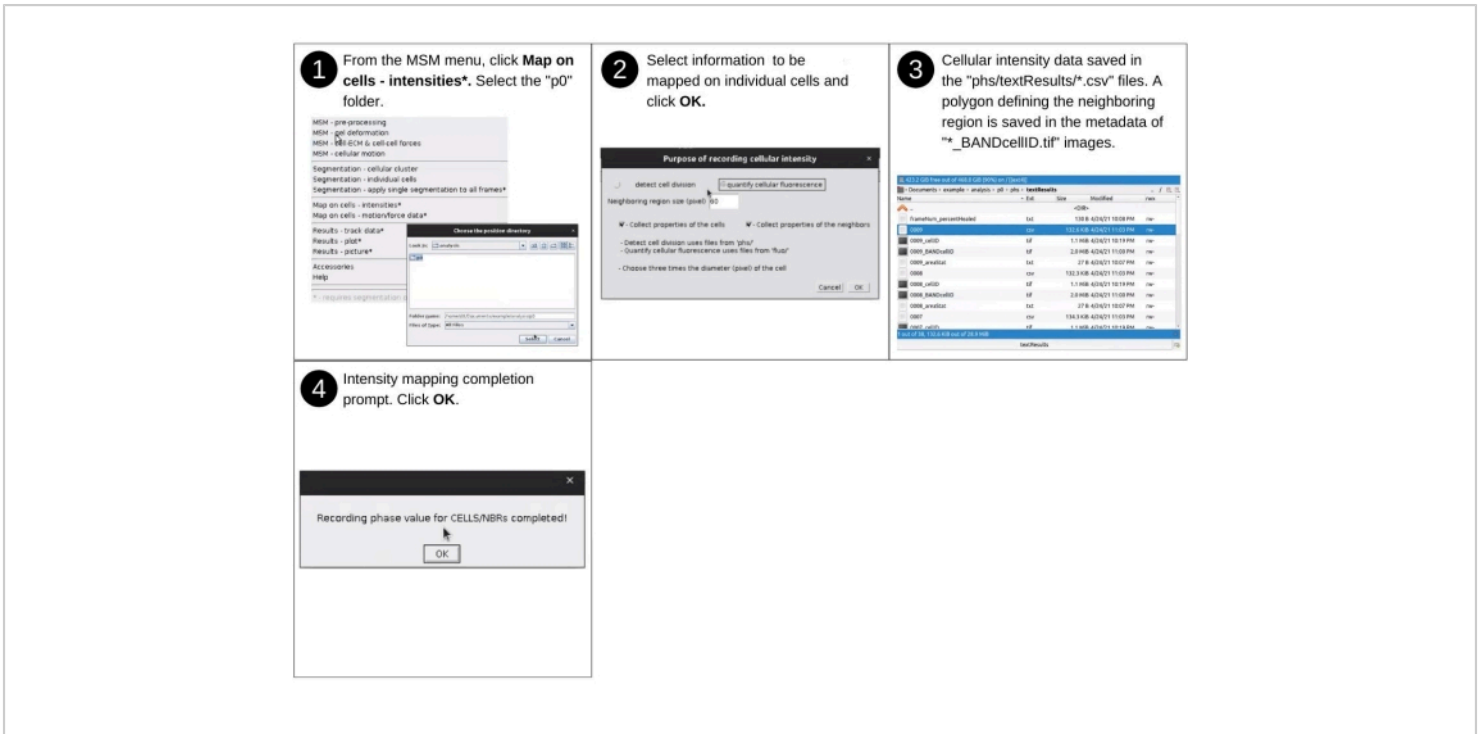


Figure 11: Mapping grid point values on individual cells - part 3. Steps to assess pixel intensities in the region within individual cells and within the neighboring region of individual cells using AnViM. The intensities assessed include transmitted light intensity and fluorescence intensity. This part maps the median value and standard deviation of the pixel intensities within individual cells and within a neighboring region of individual cells. A detailed view of step 2 is presented in **Supplementary Figure S11**. [Please click here to view a larger version of this figure.](#)

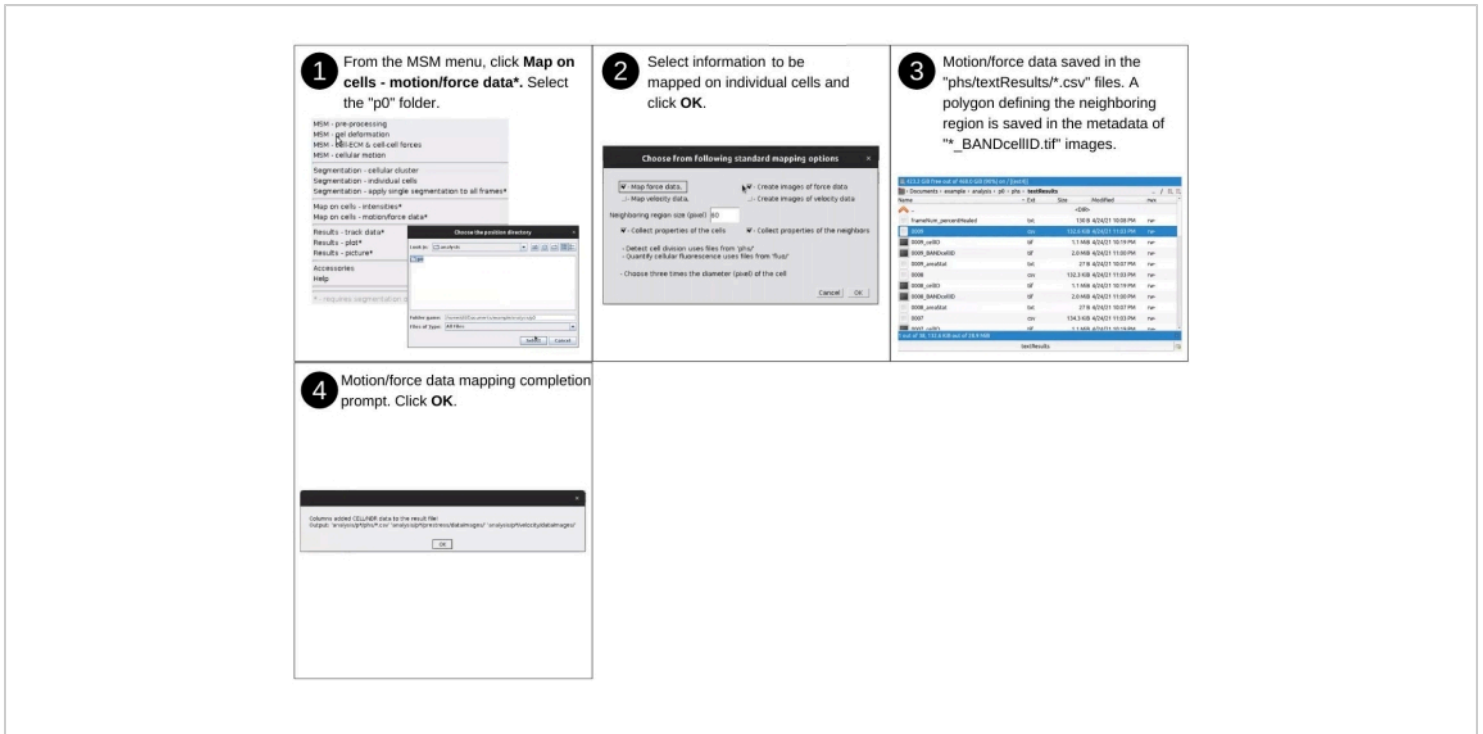


Figure 12: Mapping grid point values on individual cells - part 4. Steps to assess forces and motion properties of the grid points within individual cells and within the neighboring region of individual cells using AnViM. This part maps the median value and standard deviation of the properties within individual cells and within a neighboring region of individual cells. A detailed view of steps 2 and 3 are presented in **Supplementary Figure S12** and **Supplementary Figure S13**. [Please click here to view a larger version of this figure.](#)

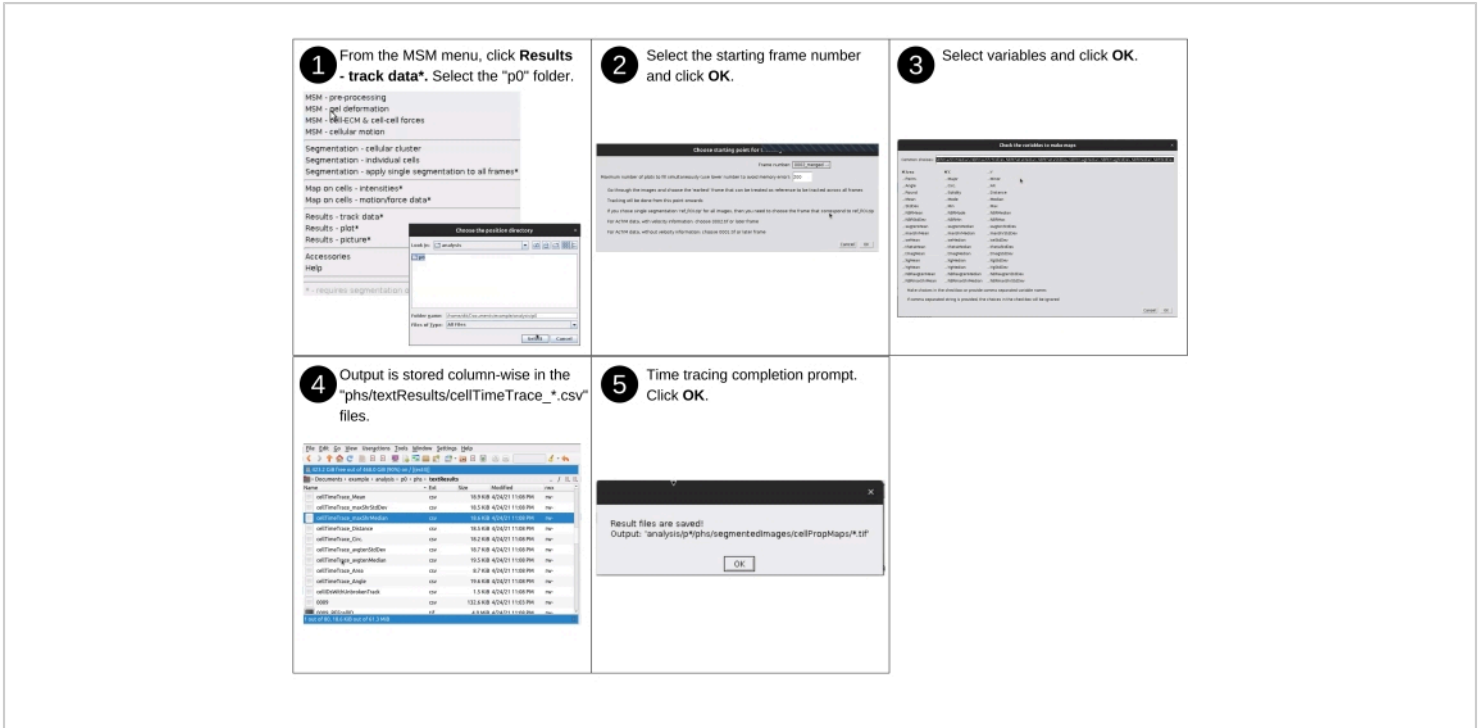


Figure 13: Visualization of results - part 1. Steps to track properties of individual cells over the entire duration of the experiment using AnViM. A detailed view of steps 2 and 3 are presented in **Supplementary Figure S14** and **Supplementary Figure S15**. [Please click here to view a larger version of this figure.](#)

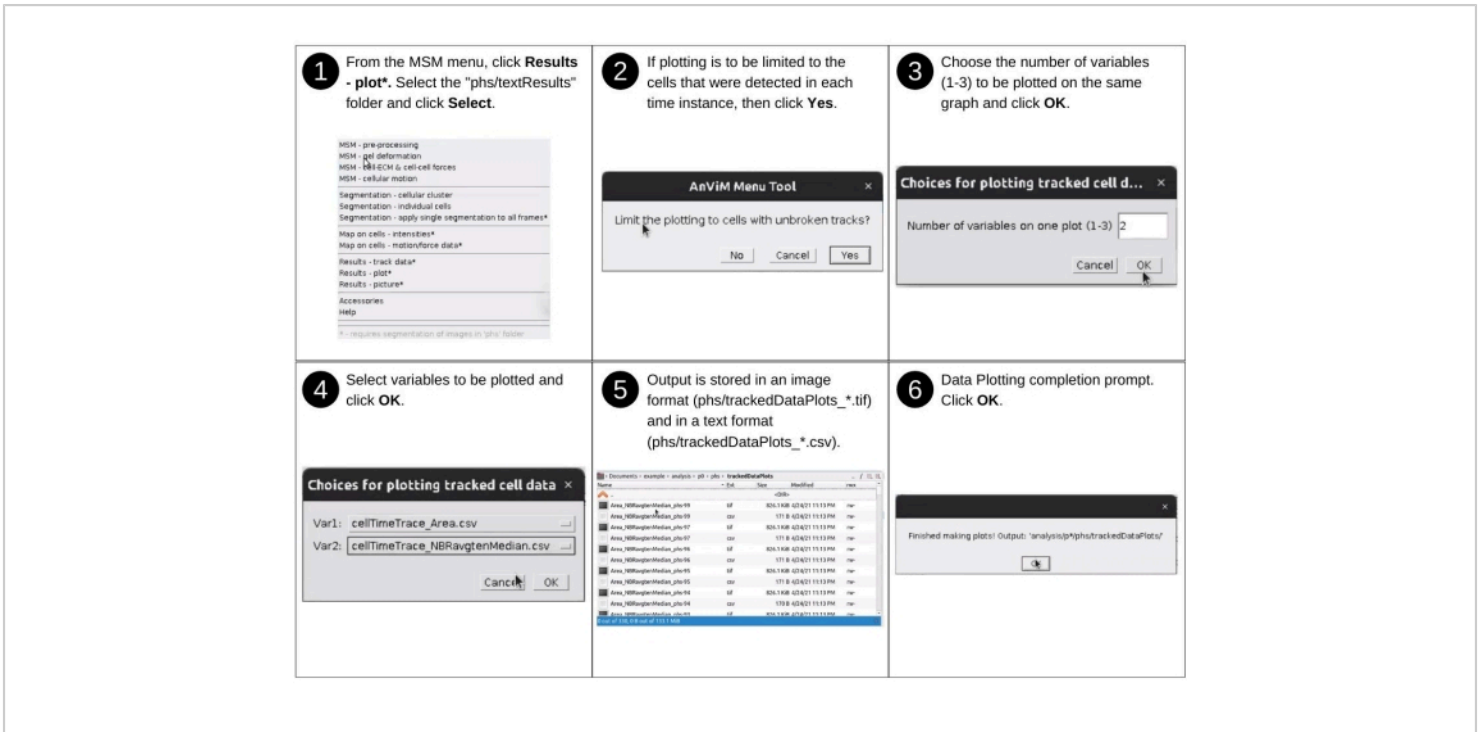


Figure 14: Visualization of results - part 2. Steps to generate time traces of the assessed properties using AnViM. The user has the option of plotting up to three properties in one graph. Time traces are generated for either all cells or only those cells for which tracking was successful across the entire experiment. A detailed view of step 5 is presented in **Supplementary Figure S16** and **Supplementary Figure S17**. [Please click here to view a larger version of this figure.](#)

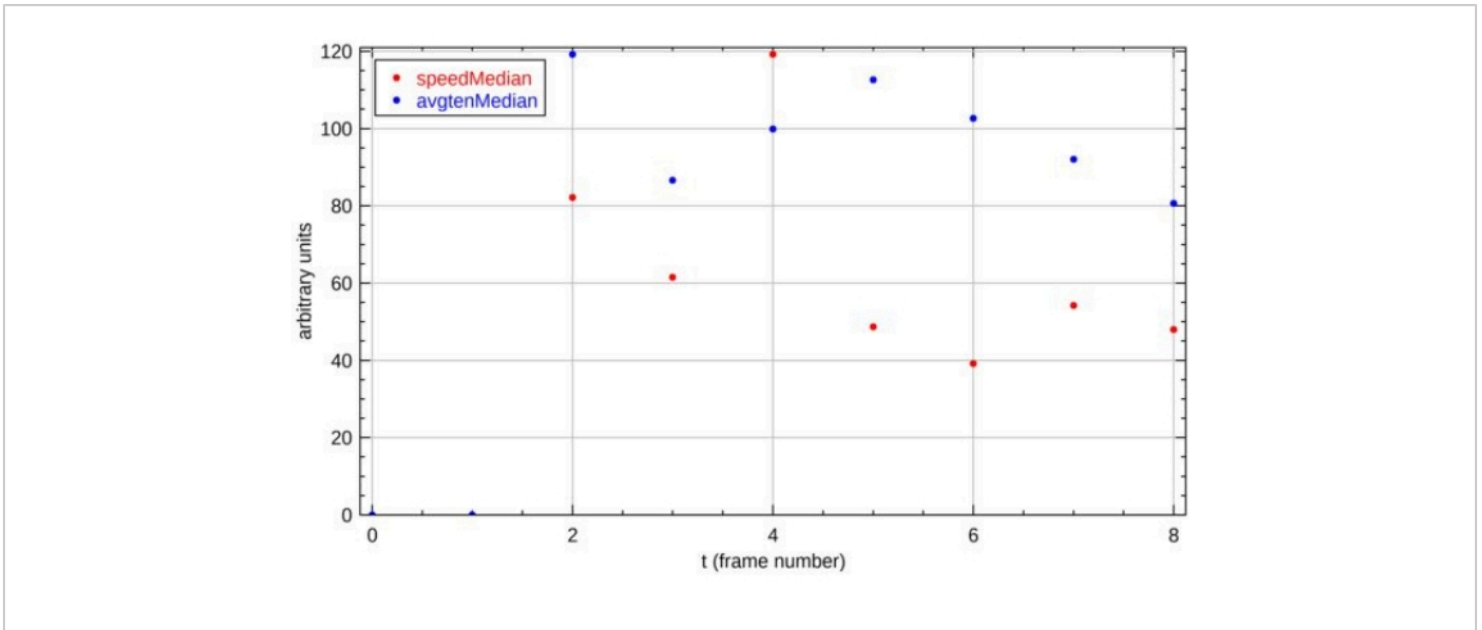


Figure 16: Time traces for cell ID 1. Two properties displayed are the cellular cytoskeletal tension ("avgtenMedian") and cellular speed ("speedMedian"). Both cellular cytoskeletal tension and cellular speed are quantified as the median value across the grid points within the cells. The two properties are plotted on the same axis with arbitrary units to visualize relationships between the assessed properties. Additional variable names are listed in **Supplementary Table S1**. [Please click here to view a larger version of this figure.](#)

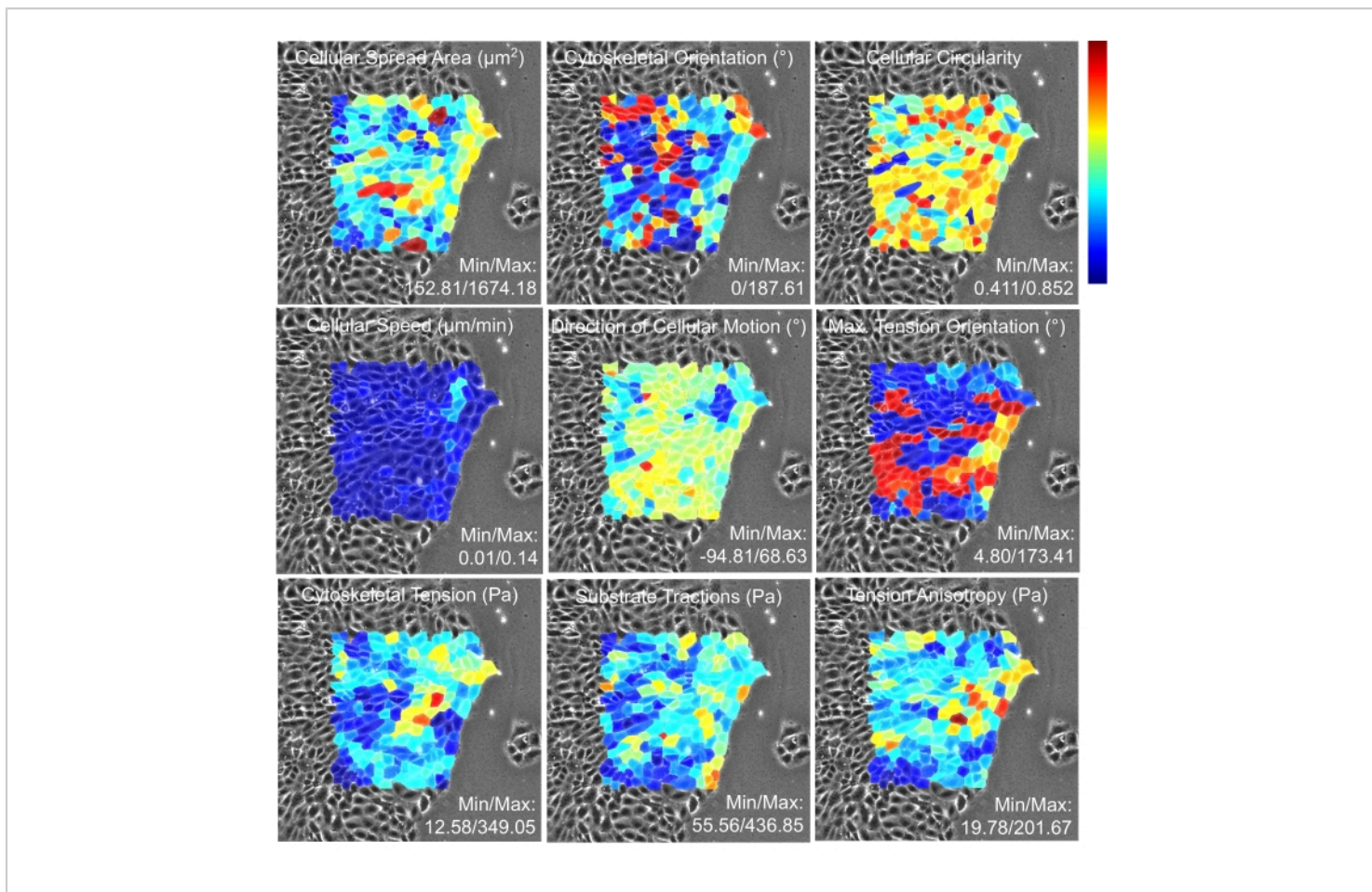


Figure 17: Heat maps of the properties of individual cells across the analyzed monolayer. Each cell is colored with the median value of the property indicated in the panel. Thus, deep red indicates the maximum cellular value in the color spectrum, and deep blue indicates the minimum cellular value across the analyzed monolayer. As described in Tambe et al., PLoS One (2013)², the cells located closer to the boundary have mechanical forces affected by unknown properties of the cells outside the image. Hence the heat map is generated for cells far from the boundary. [Please click here to view a larger version of this figure.](#)

Figure S1: Sample images of the top and bottom fluorescent bead. [Please click here to download this File.](#)

Figure S4 A: detailed view of step 6 from Figure 3. [Please click here to download this File.](#)

Figure S2: A detailed view of step 2 from Figure 3. [Please click here to download this File.](#)

Figure S5: A detailed view of step 3 from Figure 5. [Please click here to download this File.](#)

Figure S3: A detailed view of step 4 from Figure 3. [Please click here to download this File.](#)

Figure S6: A detailed view of step 5 from Figure 5. [Please click here to download this File.](#)

Figure S7: A detailed view of step 3 from Figure 6. Please [click here to download this File](#).

Figure S8: A detailed view of cell-ECM force output of step 6 from Figure 8. Please [click here to download this File](#).

Figure S9: A detailed view of cell-cell force output of step 6 from Figure 8. Please [click here to download this File](#).

Figure S10: A detailed view of step 2 from Figure 10. Please [click here to download this File](#).

Figure S11: A detailed view of step 2 from Figure 11. Please [click here to download this File](#).

Figure S12: A detailed view of step 2 from Figure 12. Please [click here to download this File](#).

Figure S13: A detailed view of the output of step 3 from Figure 12. Please [click here to download this File](#).

Figure S14: A detailed view of step 2 in Figure 13. Please [click here to download this File](#).

Figure S15: A detailed view of step 3 from Figure 13. Please [click here to download this File](#).

Figure S16: A detailed view of data files generated in step 5 from Figure 14. Please [click here to download this File](#).

Figure S17: A detailed view of a plot generated in step 5 from Figure 14. Please [click here to download this File](#).

Figure S18: A detailed view of a heat map and the file containing the range of the color spectrum generated in step 4 from Figure 15. Please [click here to download this File](#).

Table S1: A list of selected properties quantified by iTACS. Please [click here to download this Table](#).

Discussion

Adherent cells use both mechanical and chemical signals to survive, grow, and function. A wide variety of microscopy software optimizes the user experience in assessing the chemical signals through fluorescence-based imaging. However, assessment of the mechanical signals involves capabilities that are not available in the standard microscopy software. Moreover, the assessment of mechanical signals is most efficient when data acquisition is integrated with data analysis. The lack of a unified platform that meets the unique needs of mechanical signal assessment has been a major technological gap in experimental cell biology. The Integrative Toolkit to Analyze Cellular Signals (iTACS) is designed to meet this gap. The two components of iTACS, AnViM and AcTrM, equip users with the necessary capabilities to quantify cellular properties of four broad categories: forces, motion, morphology, and fluorescence/brightness. Across these categories, iTACS is currently capable of revealing more than 50 unique aspects of individual adherent cells. These aspects comprise specific properties of each broad category, including their representative value and variability across the cell (**Supplementary Table S1**). For example, within the forces, there are tensile forces across the cytoskeleton, anisotropy of this tension, the orientation of maximum tension, and shear stress across the cell-ECM interface that has a profound influence on the behavior of adherent cells^{1,3,6}.

A novel approach to examine the mechanical behavior of individual cells of a monolayer

Individual cells of a monolayer are engaged in an exchange of signals of chemical and mechanical nature³. These two types of signals are transmitted across the cellular monolayer in a different manner²³. However, the mechanical signal transmission knowledge lags behind that of chemical signal

transmission. This knowledge gap coincides with a sustained lack of simple and intuitive approaches to assess cellular mechanical signals. The novel data mapping approach described here is equipped to fill this gap. Such mapping reveals that fluctuation of intrinsic cytoskeletal tension in the neighboring region of a cell serves as relaxation, fluidization, and anchoring signals that regulate changes in cellular shape, size, and speed of the cell¹⁸. Maps of the properties of neighboring regions exhibit "multicellular subdivision" patterns where cells within the subdivision are exposed to a relatively uniform microenvironment and the cells at the boundary of the subdivision are exposed to a remarkably nonuniform microenvironment¹⁸.

Accessibility of the force measurement technology

A variety of protocols exists to make PAA hydrogels, analyze hydrogel deformation and cellular motion, and quantify cell-ECM and cell-cell forces^{1,2,7,8,9,13,14,15,18,20,21,24,25,26,27,28,29,30,31,32}. However, these developments remain out of the reach of common cell biology laboratories and confined to laboratories with engineering expertise. By automating the technical aspects of these approaches and integrating them under a unified and user-friendly platform, the goal of iTACS is to make the assessment of mechanical signals a routine activity in experimental cell biology research and education.

ImageJ allows users to develop applications using approaches that would require little or no training¹¹. iTACS is largely built using simple scripting approaches to facilitate community-driven continued development. A bulk of AcTrM is programmed using BeanShell scripts, and the bulk of AnViM is programmed using ImageJ Macros. These scripts and guidance for implementing these capabilities on the user's

microscope are available through GitHub (<https://github.com/IntegrativeMechanobiologyLaboratory/iTACS>).

Quality standardization of the acquired images

Although the elastic-substrate-based techniques to quantify physical forces in adherent cells have been developed and implemented in various labs, the protocol still lacks standardization. One area that needs standardization most is the quality of the acquired top beads images (**Supplementary Figure S1**). Significant issues arise from the drift in focus throughout the experiment. Our novel reference-image-based refocusing approach makes such a focusing an objective process. The parameters defined in the very first step of AcTrM impose necessary objective quality limits. Other standardization measures can be programmed in future versions of AcTrM.

The broad applicability of iTACS

In addition to quantifying numerous aspects of adherent cells, the iTACS structure facilitates its use for various experimental protocols and needs. AcTrM allows software-guided user self-training. High-speed imaging required by, for example, simultaneous assessment of cytoplasmic calcium fluctuations is currently limited by the speed of repositioning and refocusing hardware and is best done at one location at a time. However, the current implementation is well equipped for long-term imaging, interrupted imaging, where the sample cannot be retained on the microscope stage for the entire duration of the experiment. Since the reference images are acquired at the beginning of the experiment, iTACS enables real-time imaging of mechanical signals, opening new avenues in drug-screening applications. AnViM allows users to provide highly technical information in layman's terms. The ability to quantify a broad spectrum of cellular properties and track them throughout the experiment

constitutes critical capabilities needed to discover new intercellular communication mechanisms.

For the future development of iTACS, we have identified four focus areas: (1) enhancement of data acquisition and data analysis speed, (2) implementation of approaches to assess new cellular signals¹³, (3) development of workshops and education modules on iTACS-based cellular signal assessment, (4) development of low-cost automation solutions.

Disclosures

The authors declare no conflicts of interest.

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References

1. Tambe, D. T. et al. Collective cell guidance by cooperative intercellular forces. *Nature Materials*. **10** (6), 469-475 (2011).

2. Tambe, D. T. et al. Monolayer stress microscopy: limitations, artifacts, and accuracy of recovered intercellular stresses. *PloS One*. **8** (2), e55172 (2013).
3. Das, T. et al. A molecular mechanotransduction pathway regulates collective migration of epithelial cells. *Nature Cell Biology*. **17** (3), 276-287 (2015).
4. Vedula, S. R. et al. Mechanics of epithelial closure over non-adherent environments. *Nature Communications*. **6**, 6111 (2015).
5. Lamason, R. L. et al. Rickettsia Sca4 reduces vinculin-mediated intercellular tension to promote spread. *Cell*. **167** (3), 670-683 (2016).
6. Sunyer, R. et al. Collective cell durotaxis emerges from long-range intercellular force transmission. *Science*. **353** (6304), 1157-1161 (2016).
7. Dong, L., Oberai, A. A. Recovery of cellular traction in three-dimensional nonlinear hyperelastic matrices. *Computer Methods in Applied Mechanics and Engineering*. **314**, 296-313 (2017).
8. Nier, V. et al. Kalman inversion stress microscopy. *Biophysical Journal*. **115** (9), 1808-1816 (2018).
9. Serrano, R. et al. Three-dimensional Monolayer Stress Microscopy. *Biophysical Journal*. **117** (1), 111-128 (2019).
10. Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of Image Analysis. *Nature Methods*. **9** (7), 671-675 (2012).
11. Schindelin, J. et al. Fiji - an Open Source platform for biological image analysis. *Nature Methods*. **9** (7), 676-682 (2012).
12. Edelstein, A., Amodaj, N., Hoover, K., Vale, R., Stuurman, N. Computer control of microscopes using

- microManager. *Current Protocols in Molecular Biology*. **Chapter 14**, Unit14, 20 (2010).
13. Patel, N. G. et al. Unleashing shear: Role of intercellular traction and cellular moments in collective cell migration. *Biochemical and Biophysical Research Communications*. **522** (2), 279-285 (2020).
 14. Yeung, T. et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motility and the Cytoskeleton*. **60** (1), 24-34 (2005).
 15. Trepap, X. et al. Physical forces during collective cell migration. *Nature Physics*. **5**, 426-430 (2009).
 16. Aratyn-Schaus, Y., Oakes, P. W., Stricker, J., Winter, S. P., Gardel, M. L. Preparation of compliant matrices for quantifying cellular contraction. *Journal of Visualized Experiments: JoVE*. (46) (2010).
 17. King, J. et al. Structural and functional characteristics of lung macro- and microvascular endothelial cell phenotypes. *Microvascular Research*. **67** (2), 139-151 (2004).
 18. Patel, G. et al. Mechanical signaling in a pulmonary microvascular endothelial cell monolayer. *Biochemical and Biophysical Research Communications*. **519** (2), 337-343 (2019).
 19. Kähler, C. J. et al. Main results of the 4th International PIV Challenge. *Experiments in Fluids*. **57** (6), 97 (2016).
 20. Tseng, Q. et al. Spatial organization of the extracellular matrix regulates cell-cell junction positioning. *Proceedings of the National Academy of Sciences of the United States of America*. **109** (5), 1506-1511 (2012).
 21. Alvarez-Gonzalez, B. et al. Two-layer elastographic 3-D traction force microscopy. *Scientific Reports*. **7**, 39315 (2017).
 22. Makarchuk, S., Beyer, N., Gaiddon, C., Grange, W., Hebraud, P. Holographic traction force microscopy. *Scientific Reports*. **8** (1), 3038 (2018).
 23. Janmey, P. A., Miller, R. T. Mechanisms of mechanical signaling in development and disease. *Journal of Cell Science*. **124** (1), 9-18 (2011).
 24. Dembo, M., Oliver, T., Ishihara, A., Jacobson, K. Imaging the traction stresses exerted by locomoting cells with the elastic substratum method. *Biophysical Journal*. **70** (4), 2008-2022 (1996).
 25. Dembo, M., Wang, Y. L. Stresses at the cell-to-substrate interface during locomotion of fibroblasts. *Biophysical Journal*. **76** (4), 2307-2316 (1999).
 26. Butler, J. P., Tolic-Norrelykke, I. M., Fabry, B., Fredberg, J. J. Traction fields, moments, and strain energy that cells exert on their surroundings. *American Journal of Physiology: Cell Physiology*. **282** (3), C595-605 (2002).
 27. Saez, A. et al. Traction forces exerted by epithelial cell sheets. *Journal of Physics: Condensed Matter*. **22** (19), 194119 (2010).
 28. Deforet, M. et al. Automated velocity mapping of migrating cell populations (AVeMap). *Nature Methods*. **9** (11), 1081-1083 (2012).
 29. Polio, S. R., Smith, M. L. Patterned hydrogels for simplified measurement of cell traction forces. *Methods in Cell Biology*. **121**, 17-31 (2014).
 30. Toyjanova, J. et al. 3D Viscoelastic traction force microscopy. *Soft Matter*. **10** (40), 8095-8106 (2014).

31. Serra-Picamal, X., Conte, V., Sunyer, R., Munoz, J. J., Trepac, X. Mapping forces and kinematics during collective cell migration. *Methods in Cell Biology*. **125**, 309-330 (2015).
32. Charrier, E. E. et al. A novel method to make viscoelastic polyacrylamide gels for cell culture and traction force microscopy. *APL Bioengineering*. **4** (3), 036104 (2020).