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Microscopy tools for quantifying developmental dynamics in *Xenopus* embryos

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Summary

Early *Xenopus* embryos, and embryonic tissues isolated from them, are excellent model systems to study morphogenesis. Cells migrate, change shape and differentiate to form new tissues as embryos mature and recapitulate those same processes in tissue isolates. Both large-scale and small-scale cell and tissue movements can be visualized with a range of microscopy techniques. Furthermore, protein dynamics, fine scale cell movements, and changes in cell morphology can be observed simultaneously as multi-cellular structures are sculpted. We provide an overview of complementary methods for visualizing macroscopic tissue movements, cell shape changes and sub-cellular protein dynamics. Time-lapse imaging followed by quantitative image analysis aims to provide answers to some of the long standing questions in developmental biology: How do tissues form? How do cells acquire specific shapes? How do proteins localize to specific positions? To address these questions we suggest strategies 1) to visualize whole embryos and tissue isolates using stereoscopes and epifluorescence imaging techniques, and 2) to visualize cell shapes and protein expression using high resolution live imaging using confocal microscopy. These imaging approaches along with simple image analysis tools provide us with ways to understand the complex biology underlying morphogenesis.

Keywords

Xenopus laevis; stereoscope dissecting scope; epifluorescence; confocal microscopy; epithelia; mesenchyme; morphogenesis; cell and tissue mechanics; kinematics

1. Introduction

Early embryonic multicellular tissues provide us with a remarkable model system to understand tissue- and cell-mechanics during morphogenesis. Understanding how particular cells or tissues obtain their shape or differentiated state requires us to collect time-lapse movies using multiple imaging techniques and then analyze them to gather quantitative information. We have developed several independent preparatory and imaging protocols that can be used in complementary ways. They are outlined in this chapter: 1) visualizing whole embryos and tissue isolates (stereoscope), 2) lineage mapping and for correlation with introduced protein expression (epifluorescence), and 3) visualization of fluorescent probes for cellular morphology and protein dynamics (e.g. GFP-fusion proteins; confocal microscopy). We begin by discussing the preparatory steps and raw materials (embryo culture, handling, and care) and then discuss each imaging approach in detail.

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#equal contributions

2. Materials

2.1. Materials for imaging whole embryos and tissue isolates under stereoscope

1. Stereoscope with trinocular, video-, or camera-port.
2. Computer controllable CCD camera.
3. Computer with software to control camera, acquire images, and time-lapse sequences (we highly recommend ImageJ, a freely available software package for Windows, OSX, iOS, and Linux operating systems written and maintained by Wayne Rasband; <http://rsbweb.nih.gov/ij/>).
4. Modeling clay (Van Aken International, CA; black clay is preferred as it is less fluorescent and less toxic than other colors).
5. Glass Pasteur pipettes. One pipette with the heat-rounded tip to make depressions in clay.
6. Plastic or hair 'spears' to position and orient embryos.
7. Nitex mesh of a variety of sizes (Amazon Supply Inc., Seattle WA) to hold multiple embryos.

2.2. Additional Materials for imaging with epifluorescence-equipped stereomicroscope

1. Fluorescence-equipped stereomicroscope, non-fluorescent black background (e.g. the white/black plate used by stereoscopes),
2. Color CCD camera.

2.3. Additional Materials and Equipment for confocal microscopy

1. Confocal imaging microscope. Our group has access to a laser-scanning confocal (Leica TCS SP5, Leica Microsystems, Bannockburn IL), however, other laser-scanning as well as spinning disk confocal systems are suitable (available from Zeiss, Nikon, Olympus, and Yokogawa).
2. Immersion oil (Cargille Laboratories, Cedar Grove) is required for oil-immersion lenses (40x and 63x) for high-resolution imaging.
3. Small coverslip fragments (2 by 8 mm) are prepared in advance using a diamond pencil. We routinely store these fragments in a petri dish along with a convenient supply of silicone grease dabbed on the undersurface of the lid of the dish.
4. Prepare lint-free tissue lined Petri dishes to store culture chambers and to keep their lower glass surface clean.
5. Sparkle glass cleaner.

2.4. Materials for embryonic culture/ microsurgery

1. Commercially available tools (Fig.1A):
 - a. Silicone grease (high vacuum, Dow Chemical)
 - b. Forceps (Dumont #5 stainless steel; Fine Science Tools, Foster City, CA)
 - c. 60 or 100 mm Petri dishes.
 - d. Cover glass (small), 24 by 40 mm (12-544C, #1.5; Fisher Scientific)

- e. Cover glass (large), 45 by 50 mm (12-544-F, #1.5; Fisher Scientific, Hampton, NH) diamond pencil (retractable; Ted Pella Inc., Redding, CA)
- f. Stage micrometer (1 mm; Fine Science Tools, Foster City, CA)
- g. AmpliCap Transcription kit; (Epicentre Biotechnologies, Madison WI)
- h. Microinjection system (PLI-100; Harvard Apparatus, Holliston, MA)

2. Custom-made tools (Fig. 1B):

- a. Hair tools (hair knife and hair loop): Create the tools using 1ml syringes. Insert eyebrow hair (for hair knife, (Fig. 1B')) or baby hair (for hair loop, (Fig. 1B'')) in the syringe needle and fasten the hair in place using melted wax.
- b. Custom acrylic chambers: Use 2.5 by 5.0 by 0.5 cm acrylic block to produce an inner chamber with dimensions of 1.2 cm by 2.8 cm. Custom chambers can be milled in a suitably equipped machine shop.
- c. Silicone grease “caulking-gun”: Prepare a grease dispenser by loading silicone grease into a 5 or 10 ml syringe.

3. Solutions

- a. MBS (Modified Barth's Saline): Embryo culture media (MBS; 88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.33 mM CaCl₂, 0.41 mM (CaNO₃)₂, 0.82 mM MgSO₄, 10 mM HEPES (H3375, Sigma-Aldrich, St. Louis MO)).
- b. DFA (Danilchik's for Amy): Explant culture media (DFA; 53 mM NaCl₂, 5 mM Na₂CO₃, 4.5 mM potassium gluconate, 32 mM sodium gluconate, 1mM CaCl₂, 1mM MgSO₄).
 - i. Add 0.1% bovine serum albumin with DFA (BSA; A7906, Sigma-Aldrich).
 - ii. Add antibiotics and antimycotics (0.8 % in media; A5955, Sigma-Aldrich) fresh to inhibit bacterial growth.
- c. Calcium- and Magnesium-Free DFA: Prepared the same as DFA above except omitting CaCl₂, MgSO₄, BSA, and antibiotics.
- d. 1x MBS with 3% Ficoll (Sigma, St. Louis MO) for microinjection.

3. Methods

Before we begin the discussion of various imaging techniques, it is important to understand that there are several preparatory steps that should be completed in advance. Standard practices are followed to obtain embryos [1]. Injection strategies and microsurgies are discussed in detail previously [2] and the materials for the process are listed in the 2.4 Materials section. Preparatory steps are listed below:

Preparation

Steps for preparing live samples for imaging include preparation of mRNA, morpholinos, or lineage tracers, obtaining and culturing embryos, and microinjection and have been described previously [1]. In the following sections we describe the various imaging approaches for cells, isolated tissues and whole embryos:

3.1. Imaging whole embryos and tissue isolates using a stereomicroscope—

The first and most effective way to explore a specific morphogenetic process is to monitor movements in the intact *Xenopus* embryo or within a micro surgically isolated tissue fragment. Early developmental processes in *Xenopus* occur rapidly and can be observed either directly, or in tissue fragments. The *Xenopus* embryo develops rapidly, for instance, gastrulation takes about five hours to complete at room temperature. Advanced micro surgical techniques allow the researcher to isolate cells and tissues from surrounding tissues so that the role of non-autonomous signaling or mechanics can be explored. Such isolated cells or explants often recapitulate developmental programs of differentiation and local morphogenetic movements. Multipurpose culture chambers can provide experimental access to both gross morphogenetic movements and sub-cellular processes. In addition, pigment granules and yolk platelets in the *Xenopus* embryo provide good contrast when using side-illumination on a simple stereo dissecting microscope so that one can follow tissue and cell movements without the additional complication of fluorescent labeling techniques. In this section, we will introduce practical methods to image and analyze the morphogenesis of developing embryos and isolated tissues, and discuss tips to improve the image quality.

Protocol:

1. Prepare the embryos or micro surgically isolated tissues at desired developmental stage.
2. Transfer embryos or tissue isolates using a disposable plastic Pasteur pipettes into a small petri dish filled with culture media (DFA for isolates, 1/3 × MBS for whole embryos).
3. Position embryos or tissues with hair tools. For whole embryo imaging, there are several approaches to hold the embryos in place during development (Fig.2A, B and C; see note #1).
4. Gently fill or top-off the culture media in the dish with plastic pipette until the small petri dish is slightly overfilled. Be careful to avoid disturbing the positioned embryos.
5. Seal or cover the petri dish with large glass cover-slip by gently pressing the cover-slip from the top to avoid introducing air bubbles inside of the culture chamber (see note #2).
6. Adjust factors such as zoom, focus, brightness, contrast and color balance in live image mode, and set optimal acquisition parameters in the software (see note #3).
7. Collect single images at long time intervals or time-lapse movies (see note #4).

¹⁾ Since embryos often rotate and shift their body as they undergo large scale tissue morphogenesis, keeping embryos in place is essential for acquiring long term time-lapse sequences. To minimize the sliding of embryos during imaging, vitelline membrane can be carefully removed with forceps before positioning. Larger tissue explants can also shift position during extended time-lapse imaging. Explants can be placed within narrow slots or boxes formed from glass cover-slip fragments positioned with silicone grease.

²⁾ Covering the petri dish with a cover-slip provides higher quality optics than imaging through either an air-water interface or imaging through plastic. In addition, a tightly sealed culture chamber prevents evaporation of media that can cause dramatic shifts of imaging focus for long-term live imaging.

³⁾ Use a high contrast background surface (e.g. black paper under a light colored explant) to improve subsequent image analysis efforts. Increase the camera gain and minimize the intensity of the light source or add an IR or UV filter to the light source to reduce the heat and other hazardous effects of long-term light exposure during time-lapse movie collection. Often, the resolution or field-of-view of the stereoscope can be improved by either decreasing or increasing the magnification on the video-coupler between the camera and port.

⁴⁾ Use a manual stage to collect images at long intervals (e.g. every hour) or use a computer controlled stage to automatically collect at short intervals. Automatically collected images are aligned within the same frame-of-reference during collection whereas manually collected images can be aligned after collection.

Analysis—It is often said “a photo is worth a thousand words,” however, you will often need to reduce a photo or a time-lapse series to a small set of representative numbers. Quantitative image analysis is key to comparing the effects of treatments or defects associated with genetic lesions. We present here examples of several morphogenetic movements observable with the stereomicroscope and quantitative methods to assess their progress.

1. Measuring rates of dorsal isolate elongation or animal cap explant spreading (Fig. 2E, E', F and G). Time-lapse sequences of these explants, whose widths range from 200 to 5000 μm , change shape over one to five hours of elapsed time. Methods can be developed to automatically segment the tissue from the background. However, it is often simpler to manually identify the boundary of the explant or tissue.
 - a. Automated segmentation. Threshold the background, detect the edge of the explant, and auto-measure a region of interest (ROI) that contains the tissue but excludes the surroundings. Once the boundaries of a structure can be defined by a threshold intensity the structure can be segmented from the background using the AnalyzeParticles function in ImageJ. AnalyzeParticles can produce a list of ROIs using the ROI-Manager tool. This tool allows the user to track, maintain, save, and re-open a set of ROIs associated with a particular image sequence. The ROI-Manager provides many functions for non-destructive manipulation of ROIs and can be used to batch measure morphological properties of the ROI or the pixels contained within them.
 - b. Manual measurement. Use the mouse to draw an ROI manually. Hand selected ROIs can be stored within the ImageJ ROI-Manager then used to measure properties of the tissue such as width, perimeter, or area.
2. Blastopore closure
 - a. Kymograph produced from a time-lapse sequence collected as bottle cells form, tissues involute, and the blastopore closes. A kymograph is a 2D image which visualizes the time-rate of change of a 1-dimensional line-profile in a time-lapse image sequence. One axis of a kymograph represents the intensity along the line (e.g. the profile) and the other axis represents that intensity at different times. Timing of individual events, such as the time of blastopore closure, the formation of dorsal or ventral most bottle cells, and the rate of tissue involution can be measured from the kymograph. A kymograph can be prepared within ImageJ by first drawing the desired line-segment across the image and using the “reslice” command from the STACKS menu.
 - b. Manual tracking of individual landmarks such as cell clusters or patches of high or low pigmentation can provide rates of convergence or extension. Deformation of rectangular ROIs can provide semi-quantitative measures of convergence, contraction, or dilation.
 - c. Automated segmentation of the blastopore morphology is challenging, however, deformation maps of tissue shape changes can be estimated by following movements of discrete pigment marks or cells using texture mapping, image warping, or registration algorithms (e.g. using the UnwarpJ plugin [3, 4] for ImageJ)
3. Induced-contraction (Fig.2H):

- a. Kymograph produced from a time-lapse sequence collected as the tissue undergoes a contraction. The animal cap is a naturally pigmented epithelial tissue. Irregular patterns of pigment can be used as an indicator of tissue movements.
- b. Manual tracking of individual landmarks such as triple-cell junctions. c. Automated tracking using image warping to estimate tissue deformation.

3.2. Imaging with a epifluorescence-equipped stereomicroscope

Genetically encoded fluorescent proteins such as green fluorescent protein (GFP) and many new variants of fluorescent proteins have accelerated the use of fluorescent microscopy in developmental biology. A stereomicroscope equipped with bright-field or side-illumination uses endogenous contrast to visualize tissues and cells on the embryo; alternatively, fluorescence illumination lets the researcher visualize specific tissues, cells, or cellular structures that have been labeled with exogenously introduced fluorophores. In addition, an epifluorescence-equipped stereomicroscope allows the observation of a large field of view with an extended working distance between the sample and the objective, allowing the researcher to manipulate embryos or tissues with hand-tools such as forceps and hair tools or with micromechanical devices. An epifluorescence-equipped stereomicroscope is useful to verify the expression of fluorescent proteins and to locate labeled cells within the embryo. When tissue-specific, targeted expression is required for protein over-expression study or further high-resolution imaging, hundreds of embryos can be easily inspected and identified quickly (Fig 2 D,D' and D''). Optimally expressing embryos or tissues can then be prepared for other imaging experiments. Lastly, we often mix lineage-tracking fluorophores (e.g. fluorescent conjugated –dextran or membrane targeted GFP/RFP) with RNA-antisense morpholinos or experimental mRNA encoding proteins that block or induce a signaling pathway; co-injection of a lineage label allows rapid identification of mutant cells and tissues. In this section, we will introduce the materials, methods, and provide examples of epifluorescence-equipped stereomicroscopy.

Protocol—

1. Inject fluorophore-conjugated dextran (see note #5) or desired mRNA at one cell stage. (See preparation section above for details on microinjection). Dextran conjugates commonly used for lineage tracing may need to be treated with DEPC to remove RNase activity if present [5].
2. Incubate injected embryos to the desired developmental stage.
3. Turn on the fluorescent light source (i.e. mercury vapor lamp) attached to the fluorescent microscope.
4. Choose the appropriate filter block for your fluorophore excitation wavelength, and place the sample over the black background. Many black materials are auto-fluorescent, we have found magnetized rubber on the back of refrigerator magnets provide excellent black background.
5. 5. Select suitable fluorescent labeled embryos using a hair loop (see note #6).

⁵)Dextran conjugates commonly used for lineage tracing may need to be treated with DEPC to remove RNase activity if present [5].

⁶)The fluorescent expression levels of mRNAs are often low at early stages of development, but will increase as embryos develop and accumulate more fluorescent proteins. To improve the fluorescent signal detection from each sample, adjust the fluorescent illumination via lamp intensity. Avoid intense-long term fluorescent illumination which can bleach the fluorophore and cause phototoxic effects in cells. Caveat: over-expression of fluorescently tagged reporter constructs can perturb embryonic development. Often the best embryos will be the lowest-expressing samples!

3.3. Visualizing cellular morphology with GFP-fusion proteins with confocal microscopy

Confocal microscopy is used primarily to examine very small structures (in microns) where high resolution images and time-lapse movies can enhance our understanding of biological events that occur in morphogenesis. Confocal microscopy allows one to eliminate out-of-focus light and improve image quality [6]. This is done by detection of fluorescence light that is in or very close to the focal plane. There are several advantages of using this imaging technique as a stand-alone method or in a combination with other techniques. It enables us to collect high-resolution images of biological samples that improve our understanding of biological tissue architecture and protein locations. Collecting a set of images at fixed time-intervals allow us to understand, on a sub-cellular level, the nature of the proteins and their motion pathway to their specific locations. Collecting long-term and short-term time-lapse movies enables us to monitor tissue and cell behaviors. Also, by collecting z-stacks of images, it is possible to re-construct the three-dimensional tissue architecture by combining the set of images. Thus confocal microscopy has wide applications in morphogenesis where there is a need to image cellular and sub-cellular activities to better understand the role of different cell types and various proteins in development.

Materials/ Equipment—In addition to the materials mentioned in the “2. Materials” section above, the following items are required for an experiment involving imaging using confocal microscopy:

Preparation of chambers for long-term culture:

1. Chambers for long term culture. Custom acrylic chambers are prepared as mentioned earlier. Large cover glass is used as the bottom of the acrylic chamber. The chamber is sealed to the cover glass using silicone grease. The cover glass serves two purposes for confocal imaging, a) It serves as a transparent bottom that allows imaging, b) It serves as a thin boundary between the microscope objective and the tissue housed in the chamber and does not hinder imaging.
2. Prepare the substrate in the chamber according to your application and tissue.
 - a. Epithelial tissue isolated from the embryo needs anti-adhesive surfaces between the apical surface and the cover-slip glass surface. To reduce unwanted adhesion, coat the glass surface inside the chamber with 1% BSA in $1/3 \times$ MBS (BSA-MBS) overnight at 4° . Chambers are rinsed several times in DFA before use.
 - b. Deep or mesenchymal cells isolated from the embryo may require either a non-adhesive (see above) or an adhesive surface. To provide an adhesive surface we coat the glass surface with $20 \mu\text{g/ml}$ human plasma fibronectin diluted in 1 ml of $1/3 \times$ MBS overnight at 4° . If the experiment involves cell motility then fibronectin serves as a matrix for cells to attach and crawl. Chambers are rinsed several times in DFA before use.
3. Tissues of interest are microsurgically isolated and transferred into the chamber [7]. Tissue isolates are fixed in place under coverslip bridges, i.e. glass fragments held in place with small dabs of silicone grease on either end. Avoid excessive pressure on the fragments as they are fragile. With practice, one can make an educated guess about the amount of pressure required to keep them in place without smashing the tissues. It is good practice to position the tissues near to each other in straight lines or along a grid, so that during imaging, there is less translation of the objective and to make it easier to note the location of isolates in case there is a need to image multiple samples over time. With practice, 16 to 20 tissues can be housed in a single chamber. 4. Once the tissues are mounted in the chambers, fill the chambers

until nearly overflowing with DFA, apply silicone grease to the rim of the chamber, and seal the chamber by pressing a 24 by 40 mm cover glass into place (see note #7).

Protocol:

1. Microinject fertilized embryos with desired mRNAs at 1-cell to 2-cell development stage and culture embryos till gastrulation stages.
2. Depending upon the experiment, excise specific tissues and prepare them as described before in the “Chambers for long term culture” section above.
3. Power up the confocal imaging system.
4. Move the appropriate objective lens into place. For high-resolution imaging, oil-immersion objectives such as 40x and 63x are recommended. Apply a drop of immersion oil on the objective and then slowly place the chamber on the microscope stage. Move the focus to bring the objective toward the stage so that the oil on the objective wets the chamber bottom without creating any air bubbles in the oil.
5. Position small weights on either sides of the chamber to hold it in place.
6. Using bright-field illumination, adjust the focus cautiously. Once tissues are in focus, one is ready to collect fluorescence images.
7. Select a scan format. 1024 by 1024 pixels provides good image quality.
8. Adjust the spectral emission range and select the excitation filters. Adjust pinhole between 1 and 1.5 Airy units. The pinhole value is decided based on how wide or thick the structure is that needs to be resolve and on the level of the protein expression (see notes #8 and #9).
9. Single images at a single z-step can be captured using X-Z mode or X-Y mode (Fig. 3). Epithelial (Fig. 3A) and deep cells (Fig. 3A') can imaged as earlier stated in b. in “Chambers for long term culture”.
10. The image quality can be improved by 1) Accumulation (Line or Frame) or by 2) Averaging (Line or Frame)
11. Time-lapse movies are the best way to describe precise cell behaviors in cell and developmental biology. For capturing different types of confocal time-lapse movies, four different collection modes are: i) XYT, for collecting a series of images over time keeping a constant z-plane, ii) XYZ, for collecting a series of images as a single z-stack, iii) XZT for collecting a series of transverse images over time, or iv) XYZT, a mode combining the characteristics of XYZ and XYT to collect z-stacks over time (see note #10).

⁷)The goal is to seal the chamber with as few air bubbles as possible since these may cause the chamber to flex and images to drift over long-term imaging sessions. Keep a lint-free tissue handy to catch overflowing culture media.

⁸)While conducting experiments with confocal microscopy, use lowest possible laser power that provides good quality images. This depends on the protein expression levels as well as the type of protein that is being expressed. Low laser power ensures the least possible photo-bleaching, reduces photo-toxicity and cell damage.

⁹)Photomultiplier gain values must be kept to minimum to reduce noise. The values depend on the protein expression and background tolerance levels.

¹⁰)The researcher should follow a meaningful folder- and file-naming convention that allows easy archiving and the ability to track image information without the need to open the file. Modern operating systems allow long filenames but deep folder structures may exceed to character limit of pathnames in many applications.

Image Analysis—Images of developing whole embryos or tissue isolates and time-lapse movies of dynamically change cell shapes provide us with information on the likely events that happen in real time. Single image-panels from various time-points can illustrate the important events but they need to be supported by quantitative approaches. There are several tools including MATLAB's Image Processing Toolbox (Mathworks, Natick, MA), PAX-it Image Analysis Software (Midwest Information Systems, Inc. , Villa Park, IL), IDL (ITT Visual Information Solutions, Boulder, CO) to analyze images or time-lapse movies. In our lab, we utilize an open source tool, Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009) for image analysis.

For the purpose of this image analysis section, we will limit our discussion to analysis of the high-resolution images obtained from confocal microscopy. Image data obtained from a Leica confocal systems system can be easily converted to .TIF files. The original files from the confocal microscope are initially save and later archived in .LIF format. To convert .LIF into .TIF images for publication or post-processing we use freely available Bio-Formats plug-in for Image J. Bio-Formats (Kevin Eliceiri, LOCI, University of Wisconsin, Madison, WI) is a standalone Java library for reading image file formats including most proprietary formats from major imaging companies. We open the .LIF experiment file (includes image and information files collected during a particular experiment) in Image J using Bio-Formats and then save these individual files separately as .TIF stack files.

In the following section we offer one example of quantitative analysis of confocal image data. Previously, we have presented approaches to induce contractility in an epithelial cell sheet [8]. One approach that was discussed was to induce contraction by ablating a single cell in tightly-connected cell sheet (Fig. 3A). We observed that the rest of the cells in the field-of-view would contract when a cell is ablated. To demonstrate the contraction, we utilize complementary approaches to represent the experimental data.

1. While measuring image properties, it helps if one increases the zoom in the region of interest (Fig. 4A') to improve visualization. A short stepwise procedure is as follows:
 - a. Open a .TIF file in ImageJ.
 - b. Select the any type of selection tool (which could be rectangular, elliptical or polygon: These tools can be selected by clicking the tabs on the tools panel on Image J). Generally, it is a good practice to use rectangular tool to select the region of interest.
 - c. Draw a rectangle surrounding the region of interest.
 - d. Select IMAGE > DUPLICATE. This will provide you with a new image with only the region of interest outlined by the rectangular selection (Fig. 4A').
2. To visualize the changes in cell properties, a simple yet effective method is to create a strip kymograph of the time-lapse images (Fig. 4A"). This helps us to pictorially represent the changes in convincing way for the reader. A stepwise procedure to create a kymograph is as follows:
 - a. Open a .TIF file in Image J.
 - b. Add "Substack Maker" (authors: Anthony Padua and Daniel Barboriak) plugin from Image J website to your Image J. For this, download "Substack_Maker.class" file to the "plugins/stacks" within the Image J

folder on your computer and restart your Image J. This will add a Substack Maker tab in PLUGINS > STACKS on your ImageJ menu.

- c. Open a .TIF file (FILE > OPEN).
 - d. Click PLUGINS > STACKS > SUBSTACK MAKER. This will provide with a window to enter text. Enter all the frame numbers (separated by commas) that should part of the strip kymograph. We recommend a non-partisan method i.e. to select frames at regular intervals (Fig. 4A¹¹). Another common method is to select special frames that the author believes can convey relevant information about the experiment.
3. Intensity changes in confocal time-lapse data contain information on changing rates of protein abundance, localized assembly or aggregation, and protein activity levels. To acquire this data from image sequences requires a consistent methodology. Here, we illustrate a simple method where changes in F-actin assembly can be quantified from a series of images. In case of the induced contraction experiment, quantitative data is obtained by collecting information about the changing cell areas and the F-actin intensities (labeled with moe-GFP) within the cell. This procedure can be done by using simple built-in plug-ins in Image J as follows:
- a. Open the .TIF file in Image J.
 - b. Choose “Area” mode for measurements. (ANALYZE > SET MEASUREMENTS)
 - c. Open the ROI-Manager tool (ANALYZE > TOOLS > ROI MANAGER)
 - d. Select on the “polygon-selection” tool from the Image J menu.
 - e. Starting in the first image outline the cell or area of interest (yellow polygon is Fig. 4A) and add it to the ROI-Manager by typing the “t” key or by clicking the button that reads “ADD [t]” in the ROI-Manager.
 - f. Advance through the time-lapse by clicking “>” and outline a new area until the end of the time-window of interest.
 - g. Save your ROI set to a file that may be reopened for later analysis (see note #11).
 - h. Measure areas of the ROI cell outlines by selecting “MEASURE” in ROI-Manager. A new “RESULTS” window will now open with a list of AREA for each ROI.
 - i. Capture the dynamic changes in cell area in more cells by repeating steps (e) through (h).
 - j. Select “Integrated Density” in (ANALYZE > SET MEASUREMENTS) to record F-actin intensities.
 - k. Select the “Elliptical selection” tool from the Image J toolbar. l. Draw a circle in the region-of-interest (red circle is Fig. 4A) add it to ROI-Manager as before.
 - m. Advance through the time-lapse and repeat step (l) to record circular areas for F-actin intensity measurements.
 - n. Measure intensities within the ROI circles by selecting “MEASURE” in ROI-Manager. A new “RESULTS” window will now open. The results

¹¹)ROI-Manager can serve as a library for segmented structures within image sets. ROI data from an entire study can be archived in ROI-Manager files and used for future calculations , validation of image analysis protocols, or assessment of segmentation quality.

window contains Integrated Densities of all the recorded ROIs and can be saved as spreadsheet or text file.

- l.** Repeat the steps (b) through (n) to record cell area and F-actin intensities for as many cells as needed.
- m.** Graphical demonstration: Visual representation of the quantitative data is a critical aspect of research. Microsoft Excel (Microsoft Corporation, Seattle, WA) or Sigma Plot (Systat Software Inc., San Jose, CA) and SPSS (IBM Corporation, Armonk, NY) can be used to plot data graphically. Here we have shown an example of our study where cell areas decrease and F-actin intensities increase. The changes can be reported in real values (Fig. 4B) or as ratios (Fig. 4B'). We prefer to report both the aspects since real values are critical for the reader to make a good judgment of the experimental setup and ratios provide us with a method to compare values across experiments. The graphs constructed here are using Sigma Plot (Fig. 4B and B'). These are first steps in image analyses. More detailed analytical approaches have been discussed previously [9].

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Figure 1. Handy materials for preparing *Xenopus* embryos and tissues for imaging

A) Commercially available tools. Silicone grease (a), forceps (b), petri dish (c), large cover slip (d), small cover slip (e), and diamond pencil (f). B) Custom-made tools. Custom acrylic culture chamber (a), hair tools (b) and silicone grease “caulking gun” (c). B’) Close-up of the hair knife, and B’’) hair loop.

Figure 2. Approaches for imaging intact whole *Xenopus* embryos and large-scale changes in tissue explants

Whole embryos can be held with (A) nylon mesh, (B) a thin plastic piercing, and (C) dented clay. (D-D'') Sorting embryos with an epifluorescence equipped stereomicroscope. Embryos had been injected with mRNA encoding a dominant negative Wnt11 mixed with Rhodamine-dextran, a fluorescence lineage tracer. ((D) Control embryos (D') Dorsal targeted dnWnt (red in D'') expressing embryos) (E) Time-lapse sequences from stereoscopic time-lapse movie of developing dorsal isolate tissue. (E') Threshold based auto-segmented dorsal isolates from (E). (F) Quantified results of elongation of the isolated dorsal axis over time. The ROIs (region of interest) segmented from (E') are added to ROI-Manager and measured for morphological properties. (G) Kymograph drawn from line across the major axis of the elongating dorsal isolate. (H) Kymograph used for analyzing induced contraction. Line for kymograph and black outline shows a needle tip for adding solution (arrow head and line in right panel demonstrate the instance when the solution was added).

Figure 3. *Xenopus* embryonic cells observed using confocal microscope

A) X-Y view of a moesin-GFP labeled F-actin apical cell cortex in epithelial cells. A') X-Y view of moe-GFP labeled deep cells.

Figure 4. Image analysis

A) Original image of a moe-GFP labeled cell sheet. A high resolution view (A') of the square box inset in (A) shows three "regions-of-interest" or ROIs. A') The polygonal outline indicates the boundary of a single cell selected using the polygon tool and the circle indicates the area inside the cell where we intend to measure the protein intensity. The vertical rectangular inset is used to demonstrate a strip kymograph in (A''). Scale bars in A and A' indicate 10 μm . B) F-actin intensity (circle ROI in A') is plotted against cell area (polygon in A') along with the relationship between their normalized values (B')