

Live imaging of mRNA transcription in *Drosophila* Embryos

Carmina Angelica Perez-Romero^{1,2}, Huy Tran^{1,3}, Mathieu Coppey⁴, Aleksandra M. Walczak³, Cécile Fradin^{1,2*}, Nathalie Dostatni^{1*}

Affiliations :

¹Institut Curie, PSL Research University, CNRS, Sorbonne Université, Nuclear Dynamics, Paris, France.

²McMaster University, Hamilton, Ontario, Canada.

³Ecole Normale Supérieure, PSL Research University, CNRS, Sorbonne Université, Physique Théorique, Paris, France.

⁴Institut Curie, PSL Research University, CNRS, Sorbonne Université, Physico Chimie, Paris, France.

* Equal contribution

Corresponding authors:

nathalie.dostatni@curie.fr and fradin@physics.mcmaster.ca

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Summary

Live imaging has been used in recent years for the understanding of dynamic processes in biology, such as embryo development. This was made possible by a combination of advancements in microscopy, leading to improved signal-to-noise ratios and better spatial and temporal resolutions, and by the development of new fluorescence markers, allowing for the quantification of protein expression and transcriptional dynamics *in vivo*. Here we describe a general protocol, which can be used in standard confocal microscopes to image early *Drosophila melanogaster* embryos, in order to learn about the transcriptional dynamics of a fluorescently labelled RNA.

Key Words: MS2 system, live imaging, confocal microscopy, RNA, embryo

1. Introduction

The *Drosophila* embryo is a model system to study how cell identity is established at the right place and time during development. It was demonstrated using genetics that most of the patterning along the antero-posterior (AP) axis is set up by the expression of a set of gap genes under the control of morphogen gradients (for a review see [1]).

Quantification of gene expression during development was so far obtained from the detection of ribonucleic acid (RNA) on fixed material using fluorescence in-situ hybridization (FISH) [2]. However, the rapid development of the embryo and frequent interruptions of gene expression during mitoses result in complex dynamics that cannot be inferred from static observations. Recently, the possibility to fluorescently tag RNA in living cells using well characterized RNA targets (motifs) for specific RNA binding

proteins fused to fluorescent domains provided access to the dynamics of transcription in real time. These developments combined with advances in confocal microscopy (which allowed reducing signal-to-noise ratio and improving spatio-temporal resolution) and in genome editing to tag the RNA of interest with motifs [3] give access to time dependent measurements of mRNA transcription in living organisms during their development.

Although several systems allowing to visualize RNA have been described [3]–[6], the MS2 system is probably the most popular one [7]–[9]. It relies on the MS2 bacteriophage RNA loops that can be added to a target RNA, and its core protein (MCP) that can be fused to a fluorescent protein like green fluorescent protein (GFP) allowing the visualization of the expression of the target RNA and quantify it [4]. Recently, the MS2 system has been used to tag different genes in fruit fly development, which has helped elucidate how gene circuits interact with each other in a quantitative manner [8], [10]–[15]. For example, we used this system to study the transcriptional response downstream of the Bicoid gradient, through the expression of its target gene *hunchback* [11], [12], [16]. The MS2 system has also been combined with another RNA tagging system (PP7) to tag two different RNA with different fluorescent proteins (GFP and RFP - red fluorescent protein) in the same embryo [17]. In our analyses, MS2 signals were very difficult to observe with epifluorescence and we had to use confocal microscopy to perform optical sectioning such that we could avoid the fluorescence signal coming from the whole thickness of the embryo. With the early fly embryo, our acquisition only involves the first few microns of the periphery of the embryo and thus does not require 2-photon microscopy. Yet, this type of microscopy can be used to image transcription in tissues that require deeper imaging. Finally, light sheet microscopy can also be used even though the technical bottle neck here will be image analysis on the data collected. Here we describe a protocol to image transcription in living embryos using the MS2 system and standard laser scanning confocal microscopy.

2. Materials

2.1 Genetic material

The system requires the simultaneous expression of three transgenes:

- 1- The MS2 reporter transgene : it includes the promoter of interest upstream of an MS2 reporter cassette (in general 24 MS2 loops) fused to the sequence of the iRFP (infrared fluorescent protein) or any coding sequence for a protein that you can easily detect (to insure that the tagged RNA is eventually translated). Importantly, the position of these two sequences relative to each other and to the promoter depends on the question you ask. Briefly, a stronger MS2 signal will be obtained if the MS2 cassette is placed just downstream of the promoter (best signal-to-noise ratio) but faster dynamics of promoter bursting will be detected when the MS2 cassette is placed further away from the promoter (reviewed in [15]). Also, when designing the MS2 reporter make sure that cryptic binding sites for important transcription factors in your system are not localized in the spacers or stem loop sequences of the MS2 cassette. Finally, it is also possible to tag the 5' UTR, 3' UTR or introns of endogenous genes with the MS2 cassette in using genome editing approaches.
- 2- The MCP-GFP transgene: it expresses the MCP-GFP fusion protein at relatively low levels for increased signal-to-noise ratio. In the very early fruit fly embryo (syncytial), it was shown that an MCP-GFP without a nuclear localization signal (Δ NLS) improved detection of the specific transcription signal and reduced nuclear GFP aggregates at nuclear cycle 14 [12]. In the fly, there are available UAS-MCP-GFP lines [18] that can be combined with appropriate Gal4 drivers.
- 3- A transgene allowing the detection of nuclei: this reporter is required to identify the subcellular localization of the MS2 signal. These are for instance nucleoporin fused to RFP (Nup-RFP) to label the nuclear envelop (Bloomington # 35517) [19] or histone fused to RFP to label chromatin (Bloomington

23650) [20]. In the early embryo, the signal detected from the His-RFP transgene is very strong and regenerates during each nuclear cycle allowing for easier segmentation during image analysis. Even though it is slightly toxic for the embryo, we prefer it over other nuclear envelope markers such as maternally expressed Nup-RFP, whose signal is weaker and fades away during development.

The analyzed embryos result in general from a cross between females expressing the MCP- Δ NLS-GFP and His-RFP transgenes with males carrying the MS2 reporter. Once crossed, the resulting embryos will express maternally the MCP-GFP and His-RFP proteins and carry only a single MS2 locus, which helps for image analysis (see Tran et al., this issue). Embryos are then collected, removed from their chorion and imaged as follows using a confocal Laser Scanning Microscope 780 from Zeiss that enables the visualization of the reporter transgene in the green detection channel and the visualization of the histones in the red detection channel.

2.2 Sample Material

- 1- Appropriate fly stocks and crosses
- 2- Paintbrush
- 3- Embryo Collection Plates (ECP): Space non treated petri dishes out, so when mix is ready you can plate it rapidly. Weight 22 g of sucrose, and 14 g of bacto-agar. Add to 300 ml water and boil (using a microwave) until properly mixed and clear. While hot but clear to touch add 10ml of Pure Ethanol 100% and 5 ml of glacial acetic acid. Add 100 ml of grape juice and mix well. Add mix to the petri dishes and let dry. Store in a closed plastic box with a little water to keep the humidity at 4°C.
- 4- 10% Acetic acid solution: 10% V/V glacial acetic acid in water

- 5- Yeast paste: gradually mix active dry yeast and water until they have a texture similar to cake frosting (sticky but not runny), this will favor embryo laying. Activated yeast will tend to expand so allow space for this to happen and break the air bubbles. The yeast paste can be kept fresh for a few days covered with parafilm and stored at 4°C.
- 6- Spatula
- 7- Embryo collection cage: To make homemade egg collection chamber you can use a narrow *Drosophila* vial. Carefully cut the bottom part of the vial, making a cylinder. On one end of the cylinder, add a cotton plug to allow for aeration of the chamber yet stop the flies from escaping. Flip the flies into the egg collection chamber and add the embryo collection plate on the other end, trapping the flies in the chamber. Secure the embryo collection plate to the egg collection chamber with tape.
- 8- Double sided tape
- 9- N-heptane glue: Cut small pieces of double-sided tape and put those inside a small glass bottle (use around 30 cm of double sided tape). Add 10 ml of n-heptane in the bottle to cover the tape (under a fume hood). Let the mixture incubate overnight. The heptane should dissolve the glue from the double-sided tape, which will make the heptane sticky and is good to use until most of the glue has evaporated. Remember that heptane evaporates fast and is flammable.
- 10- Fine point Precision Tweezers
- 11- Dissecting microscope
- 12- Dissecting needle
- 13- Microscope glass coverslip (0.17 mm thickness)

- 14- Microscope slide
- 15- 10S Voltalef oil
- 16- Confocal microscope (in our setup: Laser Scanning Zeiss, LSM 780 integrated with 488 and 585 laser lines with GaASp detection)
- 17- Heating and cooling chamber
- 18- Immersion oil 518F (23°C)
- 19- Software for imaging acquisition
- 20- Phosphate Buffer Saline (PBS, pH 7.4)
- 21- Fluorescein solution: Dissolve 1 g of fluorescein into 1.5 ml phosphate-buffer saline (PBS, pH 7.4) and vortex the solution. A fluorescein precipitate (red powder) should remain in the solution, indicating that the solution is saturated (this ensures that the concentration of dissolved fluorescein in the stock solution is always exactly the same, even if solvent evaporates). If kept in the dark at room temperature, this saturated fluorescein solution will be stable for at least 2 months. This solution can then be diluted to use for fluorescence standards.

3. Methods

3.1 Collecting Embryos

Virgin females carrying the MCP-GFP Δ NLS and His-RFP are crossed with male flies carrying the MS2 reporter. The crosses must be performed one day in advance in culture vials to ensure that all females have been fertilized. Since live imaging experiments are usually carried out with a single embryo at a time, small numbers of embryos are sufficient to perform the experiment. However, it is recommended

to have at least 10 females to carry out the study. Crosses will have a good embryo yield for about 4 days. Crosses are done in a normal feeding tube (Figure 1 A), and then flies are put to lay embryos on a collection plate (Figure 1B).

3.1.1- Embryo laying

- 1-Take 10 μ l of 10% acetic acid solution and spread evenly with a paintbrush on an ECP.
- 2- With the end tip of the paint brush make a gentle grid of grooves on the ECP agar where flies will preferentially lay their eggs.
- 3- With a spatula add a small amount of yeast paste to the center of the ECP (Figure 1A).
- 4-Take out crosses from narrow *Drosophila* vials and transfer to an embryo collection cage.
- 5- Rapidly cover the end of the embryo collection cage with the prepared ECP.
- 6- Safely secure the ECP and eggs collection cage together using scotch tape (Figure 1B).
- 7- Put back embryo collection cage into the incubator (see **Note 1**).

3.1.2- Chorion removal and preparation of the coverslip for imaging

Drosophila eggs are around 500 μ m in length. They are white and can be seen by eyes on the contrasting background of ECPs. However, for chorion removal and preparation of the coverslip for imaging higher magnification is needed. Therefore, this procedure should preferably be carried out under a dissecting scope. A microscope with a low magnification objective with 2x or 5x objective can also be used.

- 1- Take 10 μ l of heptane glue with a pipette and spread it over a microscope cover slip making fine lines, let dry (see **Note 2**)
- 2-Take the ECP from the collection cage by tapping gently and rapidly cover the opened cage end with another ECP.

- 3- Using a precision tweezer or a rolled up piece of tape, take the embryos from the ECP with care and transfer them to the double sided tape slide (Figure 1C-D, see **Note 3**).
- 4- Remove the chorion of each embryo by gently touching its body, and rolling it over the double sided tape (Figure 1D-E, see **Note 4**). Removal of the chorion by hand avoids the use of bleach (bleach prevents the embryo to stick properly on the cover slip).
- 5- Once the chorion is removed, transfer the embryo to the cover slip, placing it on top of the glue line.
- 6- Place the embryo in the desired orientation for imaging (Figure 1F).
- 7- Using the length of a dissecting needle gently tap the embryo in place to flatten it (see **Note 5**).
- 8- Cover the embryos with a generous drop of Oil 10 S (see **Note 6**)
- 9- Secure coverslip into mounting device for imaging.

3.2 Setting and optimizing acquisition parameters for live imaging

Here we describe a general protocol for adjusting the acquisition parameters of a Laser Scanning Microscope to obtain optimal spatial and temporal resolution for the imaging of live *Drosophila* embryos (or, in general, large-scale dynamic systems). This protocol should be repeated at the beginning of each different imaging series (for example, each time a new type of embryo is being imaged). We used a Zeiss LSM 780 microscope and associated Zen black software to develop this protocol; however it can easily be adapted to any modern commercial confocal microscope. Table 1 and 2 contains a brief description of the imaging parameters to be optimized, and gives their typical value in our experiments as a reference. Figure 2 gives an illustration of the confocal imaging scheme and a graphic representation of some of these imaging parameters.

- 1- Select an objective with moderate magnification (e.g. 40x) and high numerical aperture (NA), which usually requires oil immersion. For example, the images shown here were acquired with a Plan-Apochromat 40x/1.4 NA oil immersion objective.

- 2- Activate the necessary laser lines and select appropriate filter cubes for the fluorophores to be imaged (in our case: GFP, maximally excited at 488nm and visualized around 520 nm, and RFP, maximally excited at 586nm and visualized around 620 nm). The emission filters should have a bandpass as large as possible to maximize fluorescence signal collection, while avoiding cross-talk between the two channels.
- 3- Set the pinhole diameter to 1 Airy unit for an optimal trade-off between resolution (both in the focal plane and along the optical axis) and signal collection. If necessary, the pinhole diameter can later be increased above 1 Airy unit to increase signal collection efficiency (and thereby possibly scan rate), at the cost of spatial resolution and sectioning power (and vice versa).
- 4- Place a representative sample (of the type that will be studied) on the microscope stage. Quickly select a laser power and detector gain that allows detecting the signal of interest (these two parameters will be adjusted more precisely at a later step).
- 5- Choose the dimensions of the region of interest (ROI) that is the size of the region in the sample that will be imaged. In general, this will need to be chosen according to your experimental question. Remember, however, that a large ROI can only be imaged at the expense of spatial and/or temporal resolution.
- 6- Next choose the number of pixels per line and per row, or, alternatively, the pixel separation (Figure 2). The Nyquist criterion can be used to determine the optimal pixel separation (see **Note 7**). As a rule, the more pixels the better the spatial resolution but the longer the acquisition time. Optimizing pixel separation is thus important, as it will greatly influence scan rate.
- 7- Choose the span (depth) and increment (separation between two consecutive images) of the z-stack for volumetric acquisition (Figure 2). Optimizing the distance between images in the z-stack will influence the number of stacks needed for imaging a ROI with a particular depth, and thus affect time

resolution. The optimal distance between images in the stack can also be estimated using the Nyquist criterion (see **Notes 7 and 8**).

- 8- The last adjustments to be made concern the pixel dwell time (or alternatively the scan acquisition time, as both are directly related). The pixel dwell time can be adjusted according to your experimental question (so that the dynamics of the process under study can be captured). Shorter pixel dwell times allow to capture faster processes and to minimize photobleaching, however this is at the cost of signal collection.
- 9- To optimize the levels of fluorescence signal detected, after a pixel dwell time has been chosen, adjust the laser power and detector gain to achieve a sufficient signal-to-noise ratio to visualize the process of interest (as estimated by eye from the acquired images). This has to be done separately for both channels. Laser power and detector gain must be adjusted together, until the best compromise is obtained. Remember that the laser power should be kept sufficiently low to minimize phototoxicity (short wavelength light is the most damaging to organisms) and photobleaching, while high detector gain can result in non-linear detector response.
- 10- If time resolution is of great importance, after all the other settings have been optimized for this purpose, a bi-directional scanning mode can be selected. This can greatly improve the time resolution, however it can also cause image artifacts when using high magnification objectives (see **Note 9**). Once all the imaging parameters have been optimized, remember to save them (e.g. as an experimental setup in the Zen Black software) in order to be able to use them again in subsequent imaging sessions.
- 11- As laser power and microscope optical alignment may vary with time, affecting the amount of fluorescence signal collected from embryos, we recommend using a control fluorescence slide to

keep a record of the intensity of the signal that can be obtained in this particular instrumental configuration (see **Note 10**). This can be used as a benchmark in subsequent experiments.

Once the imaging parameters have been optimized for a particular set of experiments, they should be saved and used consistently in subsequent experiments of the same type, allowing reproducible experiments to be used for quantitative analysis.

3.3 Live imaging of *Drosophila* embryos

Here we describe the basic procedure to record a 3D movie of a live embryo. This procedure generates a series of two-color image stacks. Each image stack is the 3D confocal image of the same region (the region of interest, or ROI) of the embryo at a different time (as illustrated in Fig. 2). Alongside with the 3D movie, we recommend performing a tile scan of the whole embryo, which will allow for the measurement of the embryo dimensions (anterio-posterior and dorso-ventral axes) and exact localization of the ROI within the embryo. Finally, we explain how to use maximum projection to produce a 2D movie that can be then exported as an .avi file and used for quick visualization.

- 1- Set the microscope incubator chamber to 23°C, and allow to equilibrate for 30 min (see **Note 11**).
- 2- Turn on the lasers to be used for excitation, and allow their output intensity to equilibrate if necessary.
- 3- Select the correct objective and carefully add a drop of water or immersion oil if required.
- 4- Load the confocal imaging parameters saved at the end of the set-up procedure described in the previous section, and record an image of a fluorescence calibration slide (see **Note 10**). Check that the signal obtained from this slide is similar to that obtained in previous experiments. A signal that is significantly lower (or higher) than usual indicates that there is an issue with the alignment or the

settings of the microscope (e.g. incorrect laser intensity, incorrect fluorescence filters, incorrect detector gain, misaligned confocal pinhole), something that should be dealt with before further imaging. Small daily variations in signal, on the other hand, are to be expected and no cause for concern. The intensity recorded for the calibration slide can then be used to normalize the intensity of the images acquired during this session, allowing comparison between the data obtained in different sessions.

5- Place the coverslip with the embryos (prepared as explained in section 3.1) onto the microscope stage. Turn on the bright field (transmitted) illumination and move the region of the sample containing the embryos (that should be visible to the naked eye) in the field of view.

6- Adjust the objective focus until the embryos come into focus.

7- Still using bright field imaging, you can quickly observe each embryo in the sample to determine which ones are in the desired developmental stage (see **Note 12**).

8- Once an embryo in the correct developmental stage has been spotted, you can rotate the coverslip to roughly orient the embryo as desired relative to the scanning axis.

9- Start live confocal acquisition to check for the presence of a fluorescence signal in the chosen embryo and confirm its developmental stage (see **Note 13**).

10- At this point, the image of the embryo may be digitally rotated to position it properly within the computer screen (e.g. for instance with the antero-posterior axis oriented horizontally).

11- Move the stage to place the region of interest in the center of the field of view.

12- Take a full sagittal plane image of the embryo by acquiring 3 images (tiles) that can be assembled to form an image of the whole embryo (Figure 3A). This will allow for the exact measurement of the embryo dimensions, as well as for the exact location of the ROI within the embryo (for example along the anterior-posterior axis). This is important for quantification and image analysis purposes. This tile scan can also be performed at the end of the experiment (see **Note 14**).

13- Chose the ROI (region of interest, Figure 3) that encompasses the area of interest in the embryo (see **Note 15**).

14- Define the depth of the region to be imaged, by choosing the position of the top and bottom planes in the z-stack (Figure 3B and 3D, see **Note 16**). The number of images in the z-stack will then be calculated according to the optimal image separation determined during the optimization step (section 3.2).

15- Select continuous acquisition, or a very high amount of imaging cycles. In this way the acquisition can be terminated based on the completion of the biological process under study.

16- Finally, start the acquisition and let the embryo develop without disturbing (see **Note 17**).

17- When appropriate, stop the acquisition and save the resulting data (see **note 18**).

18- One can perform a fast image processing of the acquired 3D movie, using maximum projection, in order to generate a 2D movie with a single image per time point (Figure 3C). This simplified version of the data can then be saved in .avi or .mov format, making it easy to disseminate or to share with collaborators. It also allows for a rapid visualization of the studied process.

Movies of transcriptional dynamics obtained with flies carrying an MS2 reporter and expressing MCP-GFP Δ NLS, as well as a nuclear marker such as His-RFP, can then be analyzed using the *LiveFly* toolbox described in the next Chapter (Tran et al., this issue).

4. Notes

1. Regular 12 hours day/night incubation cycles are important to have consistent growth and a good yield in embryo laying. Since egg laying occurs preferentially during sunset and dark periods, it can be useful to set up the incubator on an inverted day-night schedule to optimize live imaging experiments during the working hours of the laboratory.

2. Since the heptane evaporates quickly, using too much of heptane-glue will create an uneven surface of glue for the embryos to lay. It is thus important to take a small quantity of heptane-glue and spread it rapidly in a stripe like manner over the coverslip. The more even the surface, the easier it is to position the embryos and flatten them, which can influence both the quality of imaging and the development of the embryo.
3. The embryos with their chorions will easily stick to one another and to the inside or outside part of the tweezers, making the transfer easier.
4. The chorion will stick to the double sided tape. To release the embryo from the chorion, roll it gently until the chorion breaks. Once the embryo is partially released, move slightly the chorion to release the embryo completely, and using tweezers or a piece of double sided tape handled with tweezers transfer the embryo without chorion to the coverslip. A double-sided tape tip can be used to remove the chorion instead of tweezers if preferred. Removal of the chorion by hand should be a gentle process and it may take time and practice before one is able to do it properly. Also, the younger the embryos the more fragile they are and the harder it is to remove their chorion without damage.
5. Since the embryo is slightly curved, focusing on its central part may result in both its extremities being slightly out of focus, making them hard to image with high enough resolution. For studies relying on proper imaging of both the central part of the embryos and their anterior and/or posterior end, it is thus important to flatten the embryos. We have found it hard to flatten embryos without affecting their viability and expression. However, the simple step of tapping an embryo with the length (not the tip !) of a dissecting needle helps not only to glue the embryo firmly to the coverslip but also to flatten its ends.

6. The 10 S Oil is very viscous so it is hard to pipette and handle. However, it is important to cover the embryos properly and shortly after removal of their chorion to prevent them to dry. This oil is oxygen permeable such that embryos can continue to develop while under the microscope.
7. The pixel size (sometime referred to as sampling rate), d , is related to the width of the ROI (L_x) and to the number of pixels per line (n_x) through $d = L_x/n_x$. The pixel size can usually either be set directly in the software, or controlled by varying L_x (in the Zeiss LSM, this is done by changing the so-called zoom factor) and/or n_x (that is by changing the image pixel dimensions). Usually, one would use square pixels, with $L_y/n_y = L_x/n_x$. For the Nyquist criterion to be met, a minimum of two pixels must be used to image the length of an area with the dimension of the point spread function. In other words, the pixel size, d , should be no larger than the radius of the point-spread function, w (see table 2). If $d > w$, undersampling occurs, which means that spatial information might be lost. If $d < w$, oversampling occurs, which means that one is sacrificing time resolution without any gain in spatial resolution.
8. The Nyquist criterion is also relevant to the choice of the distance between two images in a z-stack, d_z . As the point-spread function is elongated along the optical axis, usually by a factor of about 5, the optimal image separation, d_z , should be around $5d_x$. However, just as for the pixel size, one can choose to trade off spatial resolution (by choosing a larger d_z) for temporal resolution.
9. To check that the bi-directional scan is not affecting your signal take a small 5 frame time series of your biological sample. Focus on an area with bright spots that remains immobile over time. Then select a single bright pixel, and check that it does not move along the scanning direction in subsequent frames, and that the image is not blurred. If problems with bi-directional scanning occur, they are usually easily seen from one frame to the next in your biological signal. If problems with bi-

directional acquisitions occur, return to the line scanning mode, which unfortunately has a much lower time resolution.

10. A saturated solution of fluorescein can be used to prepare slides with reproducible fluorophore concentration, for calibration purposes. When needed, dissolve a small amount of the saturated fluorescein solution in fresh PBS (it is very important to always use the same dilution; it is also very important that the pH of the PBS used for dilution is exactly at 7.4, as fluorescein is a pH-sensitive dye). Use this diluted solution to prepare a calibration slide (stick a coverslip on a microscope slide with heated parafilm spacers, fill the gap between slide and coverslip with the dye solution, then seal with melted wax or transparent nail polish to avoid evaporation). A fresh calibration slide (usually prepared on the same day) can be used at the beginning of each imaging session for reference: a similar signal obtained from the calibration slides before two different imaging sessions means that a quantitative comparison of the images obtained in both session can be made, while decrease in the signal measured from the calibration slide over time may indicate variations in the laser power output or misalignment of the optics (e.g. confocal pinhole alignment). If quantitative image analysis is to be done using the red channel, a similar fluorescence slide standard can be prepared, e.g. using Rhodamine. Commercial fluorescence standards such as Argolight (Argo-HM) that work for most wavelengths and are photostable for years are also available, but at a cost.

11. It is important to equilibrate the temperature of the system since changes in temperature can cause focus drift and affect image quality. A temperature control chamber that allows not only heating but also cooling is recommended, since usually heating devices are optimized for 37°C applications. However, live imaging of *Drosophila* embryos requires a temperature of 23°C. Typical incubator systems usually have difficulty maintaining the system at this temperature during acquisition, due to the additional heat emanating from the lasers. Therefore, it is recommended to place the microscope in a room kept at a cool temperature, and/or an incubator systems with both heating and cooling

capabilities, with a feedback to keep the system at a stable 23°C temperature during imaging, such as the one available from Tokai Hit (INUC-KPP Series).

12. Developmental features of the *Drosophila* embryos can be used to determine their developmental stage (their exact nuclear cycle (nc)). The earliest signal that we have been able to detect with our MS2 system has been around nc 9 or 10. A useful marker for this stage is the emergence of the polar bodies at the posterior side of the embryo in early nc 9. On the other hand, nc 14 is easily recognized by its very long duration and the size and amount of nuclei in the embryos.
13. Using a *Drosophila* strain expressing either a histone or a nucleoporin fused to RFP can help determine the developmental stage of the embryo during live imaging (by quickly inspecting the red channel), as well as the exact position of nuclei, which is important both during imaging to determine the optimal ROI, and during the segmentation step of image analysis (see Tran et al., this issue). If an embryo is found to be at an earlier developmental stage than desired, one can wait for it to develop to the right stage. It is a good idea to keep track of a few embryos at a time in the sample to anticipate which ones might reach the correct stage to be imaged. This can easily be done using a multidimensional acquisition setup to save the position of different embryos. Once one of the embryos is at the desired developmental stage, the multidimensional acquisition can be cancelled and the correct embryo selected for imaging.
14. The tile scan should cover the entire length of the embryo, including the ROI in the sagittal plane. If the embryo is horizontal, this can usually be achieved using 3 horizontal tiles. The tiles can be processed with auto-stitching, or manually stitched. It is then possible, for example, to measure the distance between the ROI and the anterior and posterior poles of the embryo. This might be important for quantification and image analysis when comparing several different embryos. The length of the embryo can also be measured using this tile scan.

15. Once a ROI has been created it can be saved and loaded, allowing to use ROI with the exact same size between experiments. The smaller the ROI, the faster the scan rate, so it is really important to make sure the imaging region is as small as possible.
16. When one is interested in imaging the cortical region of the embryo, where nuclei can be found after nc 8, we recommend to set the bottom and top image planes of the z-stack (rather than just setting the center one). The first (bottom) plane should be set above the lower membrane of the embryo and before the first nuclear signal is seen in the red channel. The last (top) plane should be set up a few microns above the first plane, after most nuclei have been observed (while moving the focus higher in the sample from the first plane). It is a good idea, however, to add one or a few planes up and down to account for focus drift during image acquisition. It is important also to use the same number of images per z-stack between acquisitions, since this will influence the scan rate, which should be consistent between experiments if quantitative comparisons are to be made.
17. At the beginning of acquisition, make sure that the embryo displays the expected features, for example that foci corresponding to nascent RNA appear in the GFP channel, and that the embryo development appears normal, e.g. by checking the nuclear signal in the RFP channel. Sometimes the chosen embryo might not develop correctly or might not develop at all, in which case another embryo will have to be chosen, or a new slide will have to be prepared, and the procedure to be repeated again.
18. The acquired image stacks can be saved in .czi or .lsm format if using the Zen software. Other formats, for example .tiff can be used with other imaging software. The *LiveFly* toolbox described in the next Chapter (Tran et al., this issue) is capable of opening any microscopy format compatible with the Bio-formats LOCI tools [21].

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Figure captions:

Fig. 1 *Drosophila* embryo collection and chorion removal. A. Setup needed for making an egg laying chamber: an embryo collection plate prepared with 10% acetic acid, yeast paste and groves; an empty feeding tube closed with a plug; and an overnight cross of flies needed for laying. B. Final setup of egg laying chamber once ready should be placed at 25° C. C. The embryo collection plate is removed from the incubator after the appropriate laying time, and eggs (marked by arrows) that are laid around and on top of the yeast paste (big white blob) are transferred to a slide (D) with a double-sided tape for removal of the chorion. E. Embryos are rolled on double sided tape (white arrow) to be released from the chorion (orange arrow). Embryos at $nc \sim 9$ without a chorion (white arrow head) are transferred to a prepared coverslip with heptane-glyce. F. The embryos are aligned and a drop of oil is added before imaging. Scale bars: 5mm (A and B), 1 mm (C), 500 μm (D and F) and 100 μm (E)

Fig. 2 Schematic representation of the confocal imaging process. The sketch shows the point-spread function at the laser focus (green revolution ellipsoid, with radius w and half-height w_z) as it scans through every pixel (each pixel is represented by a blue disk) in the ROI (width L_x , height L_y , depth L_z). The distance between two pixels is d . In this particular example, the confocal imaging results in a z-stack of $n_z = 3$ images, each with pixel dimensions $n_x = 8$ and $n_y = 6$. Also, in this example the images are undersampled ($d > w$).

Fig. 3 Live confocal imaging of a *Drosophila* embryo. A. Tile view of the whole embryo at $nc 14$, obtained from 3 adjacent tiles, auto-stitched at the end of the acquisition. The ROI within the embryo is indicated

by the dashed line rectangle. Scale bar: 50 μm . B-D. Snapshots of different views of the ROI at nc 11. B: Uppermost image in the z-stack, above the layer of nuclei. D: Bottom image in the z-stack, below the layer of nuclei. C: Maximum projection of the 23 images in the z-stack (i.e. all the images acquired between planes (B) and (D), each separated by a distance $d_z = 0.5 \mu\text{m}$). Active transcription sites are indicated by white arrowheads. Scale bar: 20 μm (B-D).

Table 1: Primary imaging parameters

Parameter	Symbol	Optimal value	Typical value
Magnification	M	Intermediate (allowing to image sufficiently large sample areas with an appropriate spatial resolution)	40×
Numerical aperture	NA	High (to achieve appropriate spatial resolution)	1.4
Excitation wavelength	λ	Intermediate (low λ generally result in higher signal levels but also more photodamage and more autofluorescence).	488 nm, 561 nm
Excitation power	P	Low (to avoid photobleaching)	3%-3.2% of laser power
Confocal pinhole diameter	D	$\sim 2wM = 1$ Airy unit (AU)	31.5 $\mu\text{m} = 0.99$ AU
Detector gain	γ	Intermediate (to achieve both a linear response and sufficient signal level)	Master gain = 700 Digital gain = 2
Pixel separation	d	$\sim w$	0.15 μm

Parameter	Symbol	Optimal value	Typical value
Image separation	d_z	$\sim 5w$	0.5 μm
Pixel dwell time	δ	Short (to allow fast imaging)	0.5 μs
ROI width, height, depth	L_x, L_y, L_z		240 μm , 100 μm , 10 μm

Table 2: Derived imaging parameters

Parameter	Relation to other parameters	Typical value
Point-spread function radius	$w \approx \lambda/(2NA)$	488nm / 561nm 0.17 μm / 0.20 μm
Point-spread function half-height	$w_z = 5w$	1 μm
Pixels per line	$n_x = L_x/d$	1200
Lines per image	$n_y = L_y/d$	512
Images per z-stack	$n_z = L_z/d_z$	20 to 30
Image acquisition time	$r > n_x n_y \delta$	0.460 ms
Z-stack acquisition time (scan rate)	$R > n_x n_y n_z \delta$	10 - 20 s

Figure 1

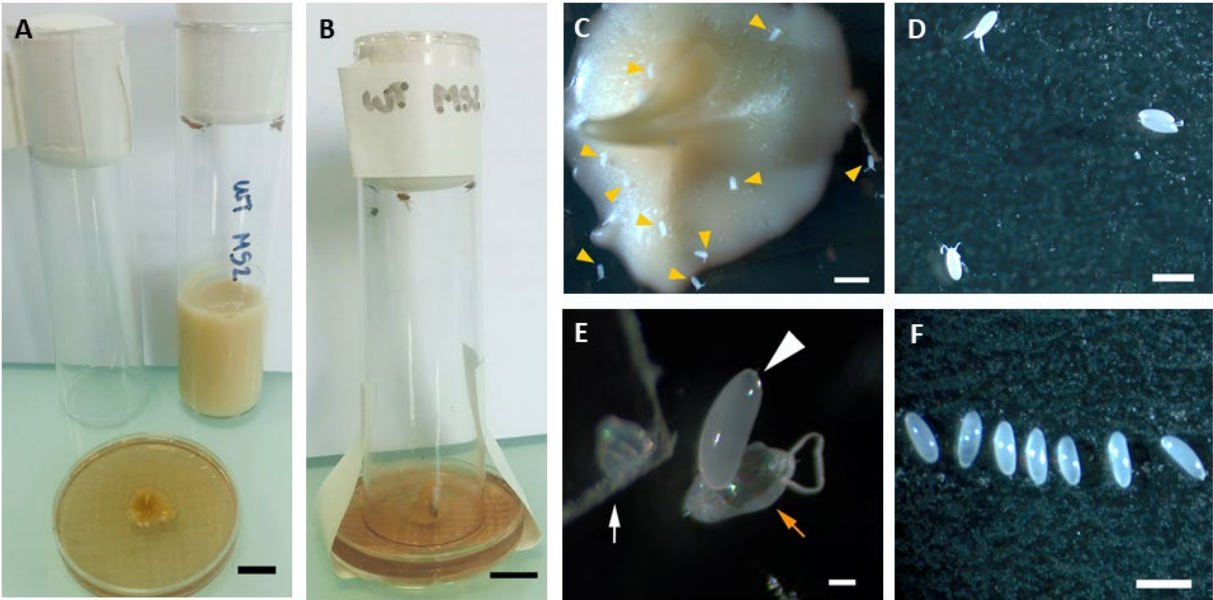


Figure 2

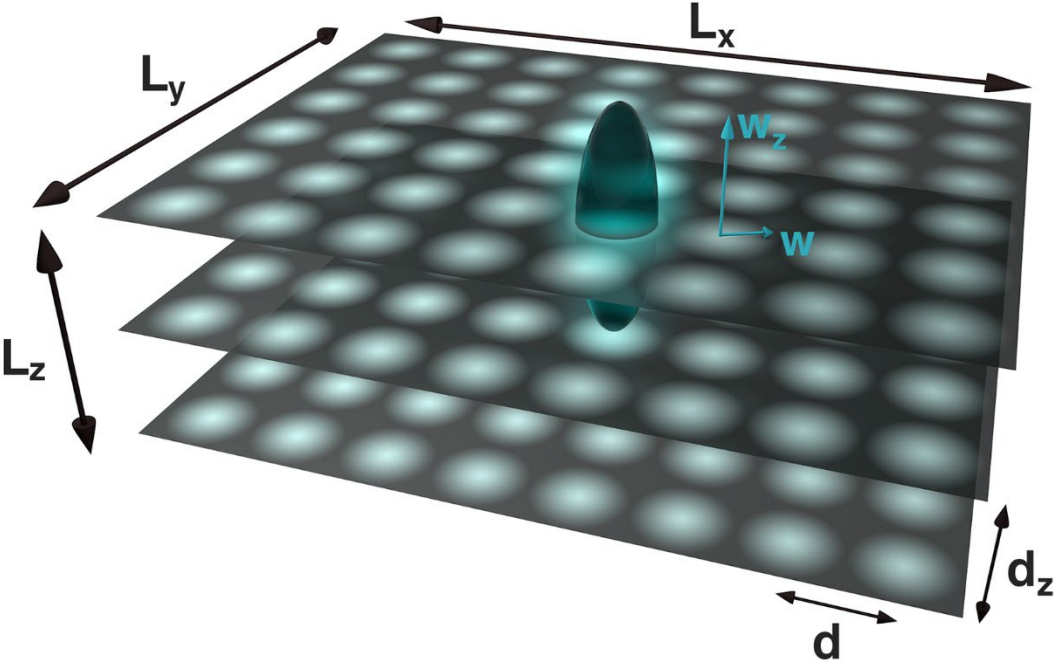


Figure 3

