Quantifying the Bicoid morphogen gradient in living fly embryos

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In multicellular organisms, patterns of gene expression are established in response to gradients of signaling molecules. During fly development in early Drosophila embryos, the Bicoid (Bcd) morphogen gradient is established within the rst hour after fertilization. Bcd acts as a transcription factor, initiating the expression of a cascade of genes that determine the segmentation pattern of the embryo, which serves as a blueprint for the future adult organism. A robust understanding of the mechanisms that govern this segmentation cascade is still lacking, and a new generation of quantitative measurements of the spatio-temporal concentration dynamics of the individual players of this cascade are necessary for further progress. Here we describe a series of methods that are meant to represent a start of such a quantification using Bcd as an example. We describe the generation of a transgenic fly line expressing a Bcd-eGFP fusion protein, and we use this line to carefully analyze the Bcd concentration dynamics and to measure absolute Bcd expression levels in living fly embryos using two-photon microscopy. These experiments have proven to be a fruitful tool generating new insights into the mechanisms that lead to the establishment and the readout of the Bcd gradient. Generalization of these methods to other genes in the Drosophila segmentation cascade is straightforward and should further our understanding of the early patterning processes and the architecture of the underlying genetic network structure.

I. INTRODUCTION

Patterning of multicellular organisms results from the interpretation of morphogen gradients by small genetic regulatory networks. The inputs and outputs of these networks are protein molecules that are synthesized by the cell and that act as transcription factors which bind to the DNA to control downstream network elements. Quantitatively mapping the relationships between inputs and outputs as well as characterizing the noise of these regulatory elements are essential for our global understanding of the patterning network. Over the past decade, a physical picture of the noise in genetic control [15] and of the global network structure that patterns the embryo [3-9] has been fairly well established. Therefore, we can use this knowledge to ask questions about the overall function and design of such networks as well as about their capacity to transmit positional information, the knowledge individual cells acquire about their spatial location within the organism. Our current understanding of such networks is mainly derived from genetics and static images of fixed tissue [10]. To fully describe the spatiotemporal regulatory interactions that determine patterning, however, a complete dynamic view is needed. Development is an intrinsically dynamic process where spatial and temporal components are intimately tied together. Characterizing the dynamics of development is important both for gaining concrete visual insights into complex developmental processes and for testing the plausibility of simple mechanisms implied by proposed models for gradient formation [11,16] and gene regulation [17,20].

Furthermore, for a fully quantitative understanding of the genetic regulation that determines the early patterning processes, we need to be able to make high precision measurements of the relevant protein concentrations in living embryos. Such measurements are nontrivial because they require high image resolution, high sensitivity, and small errorbars, which are achieved best through larger light source intensities and slow acquisition modes. However, high energies result in photobleaching of the specimen and slow acquisition times are incompatible with the inevitable concentration changes intrinsic to development. Overexposure of the embryo with light energy might interfere with the measured quantity and with the natural course of development. Finally, it is important to determine the correct correlation between the number of photons collected and the protein concentration being measured. In order to give a quantitative confidence to the measured protein concentrations, careful errorbar estimation is necessary.

II. EXPERIMENTAL APPARATUS

A custom-built two-photon excitation laser scanning microscope [21] is used for all in vivo imaging of Drosophila embryos. The microscope is comprised of a combination of commercial and custom parts, adapted to increase light collection through simultaneous detection of both epi- and transflorescence [22,23]. Figure 1 depicts the objective, stage, and condenser of the experimental apparatus. Samples are excited by light from a mode-locked pulsed Ti:Sapphire laser (Mira 900, Coherent, ~100-fs pulses at 80 MHz), whose wavelength is tuned to ~920 nm by a custom set of Mid-band filters. The laser power can be varied with an opto-electric light modulator or Pockels cell (Conoptics, model 350-80LA). Coupled scanning mirrors are used to keep the beam stationary with respect to the stage, which is capable of translation in the X, Y, and Z directions through the use of a modified Sutter MP285 micromanipulator.
tion efficiency is increased through the use of both a con-
denser and a $25\times$, 0.8 Imm Korr DIC objective (Zeiss),
whose signals are then amplified by two separate PMTs
(Hamamatsu R3986 and C6270). The advantage of utiliz-
ing this additional trans-fluorescence lies in the larger NA
of the oil-immersion condenser (NA=1.4) which provides
an increased collection efficiency resulting in an increased
signal-to-noise ratio by a factor of $\sim 2$. This configu-
ration also prevents loss of collection efficiencies due to
scattering, as the signal loss in the epi-channel sustained
with increased tissue depth is compensated by the corre-
sponding increase in the trans-channel signal, leaving the
sum of epi- and transfluorescent signals approximately
constant with variations in tissue depths [22]. The mi-
croscope is controlled by customized ScanImage software
[24], which is also used in managing the specifications of
each acquisition.

IV. LINEARITY OF ANTIBODY STAININGS

Previously, gene expression levels in Drosophila em-
bryos had been quantified using fluorescent antibody
stainings [10, 36, 37]. Such quantification relies on the as-
sumption that the fluorescence intensity levels extracted
from stained embryos and the actual protein concentra-
tions detected by the antibodies are linearly related to
the embryos natural protein concentration levels. The
Bcd-GFP fusion construct allows for a direct test of this
linearity of antibody stainings as a method of quantify-
ing relative protein concentrations. This can be achieved
by staining Bcd-GFP embryos with an antibody against
GFP (or Bcd) and simultaneously measuring the aut-
ofluorescence of GFP and the intensity of the antibody
staining. The principal difficulty here is to avoid dam-
aging the Bcd-GFP protein during the staining proto-
col. So, in order to avoid the severe attenuation of
the GFP auto-fluorescence by the usual methanol treat-
ment during the fixation process, embryos were fixed in
paraformaldehyde and subsequently hand-pealed to re-
move the vitelline membrane. Next, the embryos were
stained with an anti-GFP antibody, allowing simultane-
ous imaging of GFP autofluorescence and the intensity of the antibody
staining. Figure 2 shows comparison of these two probes at the surface and at the midsagittal
plane of a single embryo. In both cases, the fluorescence
intensity is linearly related to the protein concentration.
This proportionality demonstrates that antibody stain-
ings can be reliably used to measure relative protein con-
centrations in Drosophila embryos. The use of these an-
tibodies might be decisive in quantitatively studying the
subsequent gene network involved in the embryogenesis
of Drosophila, particularly the gap genes and the pair-
rule genes for which fluorescent fusion proteins have not
yet been developed for in vivo imaging.
Genetically modified flies are kept in various cups, whose bottoms are removable, yeasted agar oviposition plates. Typically, the flies are allowed to lay for approximately one hour after changing the oviposition plate before the embryos are harvested; however, this time can be increased to ensure a larger collection of embryos on each of the plates. It is often useful to replace the oviposition plates of various cups in staggered time intervals, each lasting 15 minutes, which will control the maximal developmental progress of each plate of embryos. This will be beneficial during the actual imaging session, as it helps to vary the time at which the embryos enter nuclear cycle 14. Harvested embryos are treated with pure bleach (8% hypochloride solution) for 15 seconds in order to remove the outer chorion membrane. After rinsing, the embryos are sorted using a stereomicroscope to select for various characteristics, such as size or developmental stage. If the time allotted for oviposition is > 2 hours, this sorting becomes important in ensuring that the embryos have not already matured passed the desired developmental stage. Once sorted, all embryos are identically oriented on an agar substrate: their anterior-posterior (head-tail) axis aligns with the y-scan of the light beam, and their dorsal-ventral (back-front) axis aligns with the x-scan. The embryos are then mounted by carefully pushing a prepared glass slide that is coated with transparent glue onto the agar. Finally, embryos are immersed in either halocarbon oil or water, depending on the employed microscope objective. While planar localization of excitation remains one of the key benefits of in vivo two-photon microscopy, the excitation within this focal plane proves non-uniform due to the decrease in laser intensity with increased distance from the focal planes center. In order to correct for this effect, a uniformly fluorescent slide is imaged to produce a flat-field correction for the later acquisitions. In a single imaging session ~ 100 embryos are mounted on a slide and viewed using ScanImage (see insert, Figure 1). By saving the embryo positions in a cycle loop, each embryo may be imaged in quick succession at low resolution with 4ms/line such that their developmental progress can be monitored. The size and density of visible nuclei provide a clear indication of the embryos current cell cycle. The completion of nuclear envelope degradation at the end of nuclear cycle 13 serves as a developmental marker that is used to ensure that each embryo is imaged at the same stage of development. Typically, images are acquired during early nuclear cycle 14, or 18 minutes after the above marker is reached. Upon reaching the desired developmental stage, the imaging configuration is changed to higher resolution with 8 ms/line and three 512 x 512 pixel frames are taken and Kalman averaged for each acquisition. Beginning with the anterior end, the embryo is imaged in three sections, with each successive image shifted 200 microns along the anterior-posterior axis of the embryo. These three images are stitched together during the data analysis to recover an image of the entire embryo in this zoomed configuration. The laser power at the sample is adjusted between ~ 5 – 40 mW (10 mW here corresponds to 5 x 10^{10} W/m² for a point-spread-function width of 0.5 µm) with the Pockels cell. In order to assess the amount of photobleaching that occurs at a given laser power, 10 high resolution acquisitions of the previously described specifications are made in quick
succession before the imaging session, and the average nuclear Bcd-GFP intensities of the images are graphed as a time series to quantify the photobleaching effect. The laser power for a given imaging session is eventually chosen such that this effect is minimized.

VI. CALCULATING ABSOLUTE BICOID CONCENTRATION

Both wild type and Bcd-GFP expressing embryos are immersed in a water solution that contains a known quantity of purified GFP molecules and imaged, as shown in Figure 3. An automated custom algorithm in Matlab (MATLAB, MathWorks, Natick, MA) identifies each nucleus within the embryo and calculates its average fluorescence intensity as follows: A mask of the embryo is obtained through a threshold that is determined by eye inspection. The original image is filtered so that each pixel within the mask is replaced by the mean intensity of a nucleus-sized disk of the corresponding pixels in the original image. From this averaged image a ring of pixels (centered on the nuclei and roughly 2 nuclear diameters wide) is created by eroding the mask. The average intensity of each pixel-segment perpendicular to the ring is determined to account for the fact that not all nuclei are located the same distance from the edge of the embryo. The algorithm, using the average values, finds the local maxima around the ring, with a minimum spacing determined by eye, to provide a rough idea of where the nuclei are. The center of the nucleus is determined as the location of the point of maximum intensity in a square of nuclear size in the averaged image centered on each peak from the ring. The intensity value for each nucleus is the average intensity of a nuclei-sized disk in the original image centered on this point. The algorithm identifies the anterior-posterior axis of the embryo and outputs a plot of the calculated intensity of each nucleus versus its position in fractional egg length along the anterior-posterior axis. The ratio of the average intensity of a nucleus-sized region in the GFP solution to the concentration of the GFP solution is used to convert the intensity values from arbitrary units into nuclear concentrations of Bcd-GFP in the embryo. To correct for background, the average intensity values of nucleus-sized regions within the wild type embryos are calculated throughout the embryo and compared across three embryos (green points with error bars in Figure 4a). This value is then subtracted from the calculated nuclear intensities to determine the absolute concentration of Bcd-GFP molecules in individual nuclei [39].

VII. MEASURING REPRODUCIBILITY ACROSS EMBRYOS

Imaging multiple live embryos using the methods described here makes it possible to measure the reproducibility of the Bcd gradient across these embryos. Initially, nuclear Bcd gradients are extracted from each embryo using the same algorithm that was used to determine absolute Bcd concentrations. These gradients are plotted together on a single graph (red dots in Figure 4a). Error bars on this graph are determined by partitioning the anterior-posterior axis into 50 bins and calculating the mean and standard deviation of the intensities in each bin (black points and error bars). The mean fluorescence background of a given image is calculated by determining the average of all intensities of the entire dataset from 90% to 95% egg length and subtracted from the data. Intensities are converted to absolute concentrations as described above. This allows us to quantify the reproducibility $r$, defined as $r = \sigma/\mu_{corr}$ where $\sigma$ is the standard deviation and $\mu_{corr}$ is the mean intensity with the background subtracted, at each location on the AP axis. In this case, the mean and standard deviation are taken over intensities from all embryos located within the given bin. The reproducibility is plotted along the AP axis with error bars determined by bootstrapping (Figure 4b) [39]. To measure cytoplasmic Bcd concentrations, a custom algorithm takes nucleus-sized disks centered at the algorithmically determined nuclei and extends each of their edges a set number of pixels (determined by eye inspection) normal to the embryo mask to create a large region around each nucleus. The cytoplasmic intensity (blue points in Figure 4a) is determined by the average intensity in this region, excluding the nucleus and a small buffer around the nucleus.
VIII. QUANTIFICATION OF ERRORS

Four main sources of measurement noise have been determined: (1) imaging noise due to the microscope, (2) nuclear identification noise due to incorrectly centering the averaging region on the center of each nucleus, (3) focal plane adjustment noise from slight differences between the imaged plane and the actual center plane of the embryo, and (4) rotational asymmetry around the anterior-posterior axis. Imaging noise is quantified by taking five consecutive images of a small section of an embryo and by calculating the reproducibility with the mean and standard deviation taken over intensities from the five images of the same embryo. Error is introduced by photobleaching of GFP due to repeated excitation. Such photobleaching effects can be controlled by imaging an embryo repeatedly and by analyzing the significance of nuclear intensity decay with each successive image acquisition. All data presented here was obtained at a laser power where the photobleaching effect was negligible ($\sim 1 - 2\%$ during the imaging process). Nuclear identification noise is obtained by artificially displacing the algorithmically found nuclear centers. For each such centers forming a $3 \times 3$ pixel area around the algorithmically determined nuclear center a new nuclear intensity is calculated using the same averaging disk. For each nucleus a reproducibility is computed with the mean and standard deviation taken at the nine locations. Focal plane adjustment noise is calculated by taking nine images, each $0.3 \mu m$ apart, with the chosen focal plane as the center image and calculating the reproducibility with the mean and standard deviation taken over intensities from the nine images of different focal planes. Error due to rotational asymmetry is estimated by comparing dorsal and ventral gradients in individual embryos to determine an upper bound on the error. The gray and black lines in Figure 4b represent the estimated contributions to noise from imaging noise and focal plane adjustment noise, respectively.

IX. OUTLOOK

The methods described here allow for imaging and quantification of maternal transcription factors in living fly embryos. Over the next decade, this approach is likely to be extended to zygotic transcription factors and other concentration measurements of the proteome, hopefully leading to a complete dynamic description of the early fly patterning cascade. Further, it should be fairly straightforward to extend this approach to related species, provided that transgenic lines can be generated to incorporate a fusion protein containing a GFP derivative. Particularly insightful would be to visualize multiple GFP derivatives of different colors in the same embryo, all tagging different transcription factors of the same small regulatory network. Cross-correlation analyses of data generated from such embryos would give us direct access to the underlying structure of the network and effectively help elucidate its design principles.

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