Three-dimensional reconstruction of gene expression patterns during cardiac development

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Soufan, Alexandre T., Jan M. Ruijter, Maurice J. B. van den Hoff, Piet A. J. de Boer, Jaco Hagoort, and Antoon F. M. Moorman. Three-dimensional reconstruction of gene expression patterns during cardiac development. Physiol Genomics 13: 187–195, 2003; 10.1152/physiolgenomics.00182.2002.—The study of the genetic regulation of embryonic development requires the three-dimensional (3D) mapping of gene expression at the microscopic level. Despite the recent burst in the number of methods focusing on 3D reconstruction of embryonic specimens, an adequate and accessible 3D reconstruction protocol for the visualization of patterns of gene expression is lacking. In this communication we describe a protocol that was developed for the 3D visualization of patterns of gene expression determined by in situ hybridization (ISH) on serial sections. The method still requires tissue sectioning, due to penetration limits of the specific staining agents into whole embryo preparations. With regard to expenditure of resources, i.e., hardware, software, and time, the protocol is relatively undemanding. Because the variation between specimens requires the visualization of multiple specimens per stage, it was decided to “do more, less well.” The current protocol, therefore, results in reconstructions of sufficient, but not the highest, quality. The use of the protocol is demonstrated on a series of serially sectioned mouse hearts, ranging from embryonic day 8.5 to 14.5. The myocardium of the hearts was identified by ISH using a mixture of specific mRNA probes and reconstructed.

mouse; embryo; heart; in situ hybridization

ESTIMATION of mRNA and protein levels is an important element in many developmental studies of gene regulation. Newly developed techniques like laser dissection microscopy in combination with quantitative PCR or mass spectrometry can be used to obtain these estimates. Because of its small size and intricate morphology, it is very laborious to apply these techniques to the developing heart. High-throughput techniques such as serial analysis of gene expression (SAGE) and microarrays provide genetic expression data, but these are devoid of spatial information. The use of staining techniques for mRNA and protein, in situ hybridization (ISH) and immunohistochemistry, respectively, enable the localization of specific mRNA and proteins in tissues with cellular resolution. Combining these staining methods with radioactive probes and autoradiography permits the calibration and subsequent quantification of the staining intensity (12, 19). For organs that are reasonably “amorphous” and composed of isotropic tissue, such as the liver, a limited number of sections can provide a quantitative estimation of gene expression gradients (J. M. Ruijter, J. Hagoort, M. M. Markman, R. G. Gieling, and W. H. Lamers, unpublished observations). However, for organs such as the developing heart, a few random sections would not suffice; the spatial distribution of the specific gene product throughout the whole organ must be mapped.

Three-dimensional (3D) reconstructions of embryonic development were originally based on computer-aided manual tracing of the organs of interest (11, 24), and the reconstructed organs were illustrated by medical artists, e.g., Ref. 27. With the advent of digital cameras, reconstruction methods based on digitized images have become commonplace. A full review of past reconstruction methods would do no justice to those researchers who had to implement their recon-
sturation protocols with the hardware and software available at that time. Recently, methods were published based on episcopic image capturing (6, 26). These epicopic methods, which acquire an image just before sectioning, enable one to accurately obtain aligned high-resolution digital stacks, based on fluorescence. Another new technique, optical projection tomography (OPT), allows the reconstruction of fluorescent and nonfluorescent whole mount specimens from transmission images acquired from different directions (20). Micro-computer tomography (μCT) and micro-magnetic resonance imaging (μMRI) can be used for 3D visualization of tissue density and water, respectively, at the microscopic level (2, 9). Still missing, however, is a protocol that is adequate for 3D reconstruction of gene-specific staining patterns in embryonic organs. Of the above mentioned methods, the OPT method is suitable for the visualization of such signals. However, the whole mount specimens themselves prevent reliable staining to occur, due to penetration problems of the staining agents, especially at later embryonic stages (20). Visualization of whole mount stained specimens with confocal microscopy (8) suffers from similar penetration problems and from decay of intensities with increasing depth (18). To accurately reconstruct patterns of gene expression, tissue sectioning remains required. With sections, the staining agent only needs to penetrate a few micrometers, which allows complete staining of the material. Another benefit of choosing sectioned material is that it enables the correlation of different labels on single or serial sections and the reconstruction of local quantitative differences in cell density and cell division (21).

The relative contributions of variance at the different levels in research show that most of the variation originates from the biological variation between specimens (7). Therefore, the expression “do more, less well” has been coined as the maxim for morphometric research. In accord with this maxim, this paper describes a 3D reconstruction protocol for patterns of gene expression. The 3D quality of the resulting reconstructions, compared with some other methods, can be considered “low,” but it is sufficient for most biological goals. For the same cost, this protocol can be used to generate multiple reconstructions instead of one single “high”-quality reconstruction. Such an approach yields the necessary information on biological variation between specimens, within the time and financial constraints of the research project. In this paper the use of this protocol is illustrated by using it to map and reconstruct the patterns of a myocardium-specific in situ hybridization (ISH) staining of a series of embryonic mouse hearts ranging from embryonic day 8.5 to 14.5 (ED 8.5 to 14.5). A detailed morphological account will be presented elsewhere (Soufan et al., unpublished results).

**MATERIALS AND METHODS**

**Sectioning and staining.** Detailed practical protocols for fixation, paraffin embedding, mounting, and sectioning of embryonic mouse tissue have been described recently (14). In short, mouse embryos ranging from ED 8.5 to 14.5 were isolated and fixed for 4–16 h in freshly prepared 4% formaldehyde in phosphate-buffered saline by rocking at 4°C. The embryos were dehydrated in a graded ethanol series, paraffin embedded, and sectioned. The thickness of the sections was increased concomitantly with the size of the embryo (Table 1). The thickness was varied to keep the resulting stack of sections for an individual embryonic heart under 200 sections, as the time required for the reconstruction also increased with the number of sections. The sections were put on a film of water on aminokylsilane-coated slides, heated to 45°C, and allowed to stretch for exactly 5 min to minimize stretch variation between slides. Myocardium was stained using a sensitive nonradioactive ISH procedure (15). The myocardium-specific ISH mix consisted of varying concentrations of five probes: α-MHC, β-MHC, cTnI, SERCA, and MLC-2v (Table 1). For each embryonic day, the ISH mix composition was optimized to yield a homogeneous staining of all myocardial parts of the heart.

**Hardware and software.** Amira (version 2.3; TGS Template Graphics Software, [http://www.tgs.com](http://www.tgs.com)) is a program for advanced visualization, data analysis, and geometry reconstruction of 3D stacks of images. Image-Pro Plus (version 4.5.1.23 for Windows; Media Cybernetics, [http://www.mediaicy.com](http://www.mediaicy.com)) is an image-processing program and offers a wide variety of image-processing and analysis functions. These functions can be strung together into custom-written programs for batch processing of images. These batch programs will be referred to as “macros” in this paper. The desktop computer used for this research operated under Windows 2000 ([http://www.microsoft.com](http://www.microsoft.com)) and contained a 2-GHz Pentium 4, CPU, a 64-MB GeForce3 Ti 500 graphics card, 512 MB of internal memory, and a 40-GB hard disk.

<table>
<thead>
<tr>
<th>Embryonic Day</th>
<th>Thickness, μm</th>
<th>Microscope Magnification</th>
<th>No. of Sections</th>
<th>α-MHC, ng/ml</th>
<th>β-MHC, ng/ml</th>
<th>cTnI, ng/ml</th>
<th>SERCA, ng/ml</th>
<th>MLC-2v, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED 8.5</td>
<td>8</td>
<td>10×</td>
<td>41</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>ED 9.5</td>
<td>8</td>
<td>5×</td>
<td>76</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>ED 9.5</td>
<td>8</td>
<td>5×</td>
<td>62</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>ED 10.5</td>
<td>5×</td>
<td>127</td>
<td>168</td>
<td>250</td>
<td>250</td>
<td>250</td>
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</tr>
<tr>
<td>ED 10.5</td>
<td>5×</td>
<td>127</td>
<td>153</td>
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</tr>
<tr>
<td>ED 14.5</td>
<td>2.5×</td>
<td>101</td>
<td>101</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>0</td>
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</tr>
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α-MHC, α-myosin heavy chain (13); β-MHC, β-myosin heavy chain (13); cTnI, cardiac troponin I (1); SERCA, sarcoplasmic-endoplasmic Ca²⁺-ATPase 2a (16); and MLC-2v, myosin light chain 2v (17).

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Image acquisition. The stained sections were digitized with a peltier-cooled digital monochrome CCD camera (Photometrics; 1,317 \times 1,035 pixels, 12 bit, linear over its whole dynamic range) attached to a Zeiss Axioshot microscope, with normal bright-field illumination. Color images were obtained by using a liquid crystal tunable filter (MicroColor, RGB-MS; Cambridge Research & Instrumentation, http://www.cri-inc.com), placed as a slider in the microscope. By capturing the same image using a red, green, and blue exclusive filter, color images are obtained (RGB).

The images are immediately processed by a custom-written macro in a camera control and image-processing software program (PMIS Image Processing Software; GKR Computer Consulting, http://www.gkrcc.com), which applies a flatfield correction and reduces each channel from 12-bit to 8-bit, effectivly storing a 24-bit color image (Fig. 1A) for each section (Fig. 1, “image acquisition”). Additionally, a 2 \times 2 binning procedure is applied, which resizes the 1,317 \times 1,035 images to 658 \times 517 pixels. This size reduction also speeds up the entire process as each image is reduced from 4 MB to 1 MB. The resizing of the images only leads to a minor loss of contrast with respect to the background. Amira was set to exclusively import the red channel (R) of the 24-bit stack (RGB). In our approach each individual stack is aligned in an iterative procedure. During the procedure, two variables of the alignment module were tuned to make optimal use of the patterns contained in each image: 1) the range of gray values that are included in the alignment algorithm and 2) the step size (in x- and y-direction) that is used in fitting one image on another. In the first cycle the step size is set to the maximum, and the range of gray values is set to include both the background tissues and the hybridization signal, resulting in a rough alignment in which the entire embryo serves as an external reference for the heart. Following the first cycle, the stack is inspected, and faulty aligned images are corrected manually. Then the stack goes through another two cycles; once on the hybridization signal with a medium step size and once more on the hybridization signal using the lowest step size. After alignment of the red channel, the original stack of 24-bit color images is loaded into Amira, and the translational and rotational parameters from the red channel are applied to the 24-bit stack, effectivly obtaining an aligned stack of 24-bit color images (Fig. 1, “alignment”).

Thresholding. Each individual image of a stack was then processed in Image-Pro Plus by a custom-written “dynamic threshold” macro. This dynamic threshold macro converts the 24-bit color images into binary images containing only the signal of interest (Fig. 1B), regardless of the between-section variation in background and signal intensities (Fig. 1, “automatic thresholding”). The dynamic threshold applied by this macro is based on an automatic analysis of particle and area profiles within the image. A detailed description and validation of this dynamic threshold procedure will be published separately (A. T. Soufan, J. Hagoort, and J. M. Ruijter, unpublished results). In short, the image is split into several classes based on the behavior of a parameter that is extracted from the image using the selected area and particle number at different threshold values. This results in a stack of aligned binary images displaying the myocardium, which is examined to evaluate the specific signal isolated by the macro. In sections in which the macro was not successful, the signal was isolated manually (Fig. 1, “manual thresholding”). If less than 80% of the sections were thresholded as intended, then the current stack was discarded and a new set of sections was prepared and digitized (Fig. 1, “% success?”).

Lumen labeling. To label the lumen of the heart, the stack of binary images was again processed in Image-Pro Plus, using the custom-written “label lumen” macro (Fig. 1, “label lumen”). This macro consists of an automatic and an interactive part. First, enclosed structures are automatically labeled in a separate color, thus effectively labeling the cardiac lumen. Second, the macro enables the manual closure of “spaces” that were not fully enclosed by myocardium, but by nonmyocardial tissue of the heart. The user places caps to close these structures in each section. The lumen macro converts the binary images into 8-bit gray-scale images with four gray values in which a value of 0 (black) stands for the background, 127 for lumen, 150 for user-drawn caps, and 255 (white) for myocardium. The macro also applies a stair-stepping and surface breakage present in the images, dust particles, using Amira’s limited, but sufficient, interactive editing tools. At this stage, sections that were beyond repair were deleted from the stack and replaced by combinations of adjacent sections. Effectively, this repair procedure is performed in 2D (Fig. 1, “2D correction”), but, using Amira, 3D information of adjacent images can be used to guide the process.

Labeling. To visualize the stack in 3D using Amira, it is necessary to convert the 8-bit gray-scale stacks into another format, the so-called LabelField file, which is closely related to uniform scalar fields. This conversion was done using the LabelVoxel module of Amira. In this LabelVoxel module, three thresholds were placed (at 100, 130, and 180), effectivly separating the four gray values defined in the lumen labeling section (Fig. 1, “labeling”).

After the conversion, the labels are enhanced using tools contained in the segmentation editor. Besides manual editing, a smooth step in 3D is implemented to compensate for the stair-stepping and surface breakage present in the z-direction, which are inherent to sectioning. The result is a LabelField file containing the boundaries of each individual gray value in 3D.

Surface conversion. The LabelField file containing the structures of interest was then converted into a 3D scalar field, using the generalized marching cubes (GMC) module of Amira (Fig. 1, “surface conversion”). The module compuates a triangular mesh of polygons of the interfaces between different structures in a LabelField file. This is done by means of a GMC algorithm. The resulting surface file can be graphically drawn, e.g., rendered (Fig. 1D). This render is already adequate for judging the shape of the heart (Fig. 1F) or measuring the volume of the individual structures. The surface render can also be manually rotated and scaled. Upon inspection of the result in 3D, holes may be visible in the structures (Fig. 1, “holes?”). These are often the result of...
misalignment between sections in the $z$-direction or by warped sections. Holes that appear to be artifacts are manually closed by making the necessary alterations in the 3D LabelField file (Fig. 1, “3D correction”).

**Volume conversion.** To enable the user to virtually cut the reconstruction and obtain virtual cross sections, it is necessary to convert the surface file to a volume file, as a structure in the surface render is simply a hollow shell (Fig. 1D). The conversion
of a surface file to a volume file is done using the TetraGen module, which creates a volumetric grid of tetrahedrons (Fig. 1, “volume conversion”). The resulting volume render can now be virtually sectioned correctly in any direction (Fig. 1E).

RESULTS AND DISCUSSION

Reconstruction results. The myocardium and the cardiac lumen of a developmental series of mouse embryos (ED 8.5–14.5) was mapped and reconstructed (Figs. 2 and 4) using the protocol described in the MATERIALS AND METHODS. The myocardium of the heart is defined as the structure that stains with a mix of myocardium-specific mRNA probes using the nonradioactive ISH method. A stage-dependent mix of probes was used to guarantee an evenly stained myocardium from the posterior to the anterior pole of the heart at each stage.

The repeatability of the current protocol is effectively illustrated by the series of reconstructions shown in Figs. 2 and 4, whereas the similarity of the duplicate hearts at ED 9.5 and 10.5 shows the reproducibility of the protocol.

Sectioning vs. whole mount methods. In literature, several methods for reconstructing embryos have been described (2, 6, 8, 9, 20, 22, 26, 28). These reconstruction methods can be separated into two groups: invasive (the tissue is serially sectioned and stained) and noninvasive (the embryos are stained whole mount). Comparing the invasive methods against the noninvasive methods, one has to conclude that the main advantage of the invasive method is that gene expression patterns can be determined unambiguously. Sectioning allows reliable staining of the material because the staining agent only needs to penetrate a couple of micrometers. Undoubtedly, whole mount methods using very small tissue samples also do not suffer from penetration problems, and indeed none are described to occur in embryos of ED 10.0 (8). However, the noninvasive methods suffer from penetration problems at later stages of development, thus creating an uncertainty in the visualization of the signal, as reported for OPT on an ED 11.5 mouse embryo (20).

With regard to the 3D quality, the noninvasive methods are undeniably superior to invasive methods. The main difference between the two groups of methods lies in the resolution and quality of a stack in the z-direction. The z-resolution in the noninvasive methods is, most often, identical to the xy-resolution (isotropic sets). In the invasive method, the z-resolution is directly linked to the section thickness. Thus the z-quality is impaired by the section thickness and reduced even further by the imperfect alignment and morphological deformation introduced by sectioning. The latter is not entirely true for the episcopic image-capturing methods (6, 26), where image acquisition is performed before sectioning of the material. However, because the staining occurs before cutting, a tradeoff is made between the high z-quality and the poor penetration of the stain. On the other hand, the xy-resolution of the invasive methods is still higher than most of the whole mount methods. It is true that this does not compensate for the low z-resolution (thus 3D quality), but the high xy-resolution is a requirement for many molecular and embryological studies, e.g., studies that aim to assess the relationship between cell density and division (21) and patterns of gene expression during development.

Required resources. Most papers do not discuss all the required resources, i.e., hardware, software, and time, in much detail. The hardware requirements for the protocol presented in this study are very lenient. No custom-made hardware has been used: one only
needs to be able to digitize stained sections and have access to a decent desktop computer. Other methods, such as μMRI, μCT, and OPT (2, 9, 20, 28) require highly specialized and dedicated equipment and software. The software required for the current reconstruction protocol is also not very “heavy.” The two software programs used were designed for end users (e.g., they do not require programming skills) and can be purchased at the sites described in the MATERIALS AND METHODS. The custom-written macros to automate the “thresholding” and “label lumen” steps of the protocol are available on request.

Software. The initial protocol made use of five programs, which was considered unwieldy as it required several conversions of image formats and made the digital reconstruction unnecessarily time-consuming. To alleviate this, a reduction in the number of programs was required. A variety of programs was tested to obtain a slim, yet versatile, package of software, that still contained all the required visualization and image-processing tools. The final package consists of only two programs: Amira and Image-Pro Plus. Both programs have a solid base of developers, and updates are being released regularly. To further decrease the required amount of time, we automated as many steps as possible. This was successfully done for two steps: the thresholding step and the labeling of the lumen. The thresholding macro, used for converting color images (24-bit) to binary images (1-bit) by placing a dynamic threshold (Fig. 1, “automatic thresholding”), operated as intended for most stacks. Although the macro was written to compensate for biological and histological variation between sections (images) and between embryos (stacks), an average of 1 in 20 images still needed intervention by the user. A plethora of image “traits” could result in faulty automatic thresholding. For instance, the atria could display an overall lower signal than the ventricles, which resulted in the signal in the atria being identified as background and omitted from the binary image. The “label lumen” macro, included as an optional step in the protocol, also worked for most images. Enclosed structures were automatically identified and given a separate color to discriminate them from background and myocardium. Falsely labeled areas did arise in the outflow region in between the large arteries and in those areas of the heart were the “outside” is enclosed by myocardium, as might occur in the atrioventricular sulcus region. These false lumen areas had to be removed manually.

Accessibility of the protocol. Initially, a reconstruction took about 7 days to complete. The implementation of a standard protocol, custom macros, and the revision of the software package reduced the time required for a reconstruction to 2–3 days. In both cases the histological steps (also 2–3 days) are not taken into account. However, reconstruction is riddled with pitfalls caused by the amalgamation of biology with computer technology. To smoothly deal with these pitfalls,
experts from both fields should be close at hand during the reconstruction process.

After more than 20 reconstructions, a 3D reconstruction protocol was developed that demands few resources and is suitable for the visualization of gene expression in embryonic organs. All that the reconstruction method described herein requires is a stack of stained sections as input, an image-capturing device, a personal computer, two software programs, and two downloadable macros.

Reconstruction quality and biological variation. As mentioned above, the resolution in the z-direction of the reconstructions obtained by using the protocol is “low” compared with some other methods. This is mainly caused by morphological deformation, which is inherent to sectioning. Correction of deformation using external marker-based automatic congruencing (22)

Fig. 5. Reconstructions of the lumen of the hearts shown in Fig. 2. Duplicate reconstructions are shown for ED 9.5 and 10.5. This series clearly shows the formation of atrial (smooth walled) and ventricular (trabeculated) lumen from the outer curvature of the heart tube.

Fig. 6. An ED 9.5 lumen. The primary heart tube is shown in purple, and the left and right ventricles (LV and RV) and mostly obscured left and right atria (LA and RA) are depicted in gray. These reconstructions support the ballooning-heart model (3, 5) in which the chambers are thought to “balloon” out of the primary tube, as opposed to the textbook model of heart development, the so-called segmental model.

Fig. 7. A reconstruction of an ED 13.0 rat heart, stained with a radioactive in situ hybridization for sarcoplasmic-endoplasmic Ca\(^{2+}\)-ATPase (SERCA). The amount of bound radioactivity is expressed in counts per minute per square millimeter (CPM/mm\(^2\)) and is a quantitative measure for the SERCA mRNA concentration in the various compartments of the heart. To facilitate interpretation, only the quantitative data in the plane of sectioning is shown; the outer surface is shown in gray.
has not been attempted, as it is almost impossible to place external markers close enough to the embryonic heart. Although it is possible to manually correct for deformation and to obtain a technically “perfect” reconstruction (25), this strategy has not been implemented in the current protocol, because this would not result in a biologically better reconstruction. Rather than investing the available amount of time and money in a single technically perfect reconstruction, we opted for multiple reconstructions. This chosen scheme would still give us the required information with regard to the morphology, but, most importantly, also insight into the biological variation present in the developing heart. A technically perfect reconstruction, however, would give us no insight into the variation present between different biological specimens. This rationale is in accordance with the recommendation “do more, less well” that is given for morphometric measurements (7). The consequence of this choice is that definite statements with regard to cellular details cannot be made based on the resulting reconstructions. The quality of the reconstructions (Fig. 2), however, is still sufficient to fulfill most biological goals. When one wishes to discuss details, one has to refer to the original sections. The reconstruction then allows the morphologist to place these details in their proper spatial context. Also, the reconstructions can be used to locate the proper position of individual sections from other experiments (Fig. 3). To this purpose, a complete series of reconstructed hearts (ED 8.5 to ED 16.5; 2–3 reconstructions per time point) will be made available. For details, E-mail to bioinfo@amc.uva.nl, subject: 3Dmouseheart.

Biological result. The appearance of the reconstructions shows that hearts from the same embryonic day are virtually identical (Fig. 2). Two or three reconstructions of the same embryonic day will probably suffice to obtain a representative reconstruction of that developmental stage. The morphology of the reconstructions closely resembles that seen in whole mount-stained hearts (Fig. 4). The lumen of the hearts was labeled and reconstructed as an optional part of the reconstruction process (Fig. 5). The main difficulty in understanding the developing heart lies in the conversion of the initially single-circuited blood flow into a double blood flow in parallel arrangement. Where in myocardium reconstructions the intricacy of this blood flow is still obscured, the virtual lumen casts expose the blood flow and will therefore add to the insight of the heart development in its entirety. These casts also support the ballooning-heart model (3, 5) as these reconstructions clearly show that the ventricles “balloon” out of the primary heart tube, as is shown for the lumen of an ED 9.5 heart (Fig. 6).

The current protocol can also be used for the 3D reconstruction of the expression patterns of heart-specific genes and transcription factors determined by qualitative (15) and quantitative (19) ISHs. The latter is demonstrated by applying the protocol to a series of sections stained with a radioactive ISH in which the staining intensities vary over a wide range of values. Since these staining intensities are directly related to the amount of probe bound to the section (19), the resulting reconstruction (Fig. 7) shows the quantitative distribution of mRNA concentrations in the heart.

Volumes obtained from the myocardium are plotted in Fig. 2. The volume data extracted from the duplicate hearts differ by a maximum of 10%. This “preliminary” biological result indicates that the biological variation may not pose a problem for generalized use of this series. Note that these volumes have been obtained using the Cavalieri principle and are therefore unbiased estimates of the myocardium volumes (10). Shrinkage, which is known to occur during fixation and embedding, has not been corrected. However, it is reasonable to assume that the relative values of the myocardium follows the plotted trend. Therefore, these volumetric data of the developing heart structures can be used in mathematical and functional models of heart development. One can calculate from these data that the myocardium volume increases 100 times in 6 days (between ED 8.5 and 14.5). Assuming that all cells present in the heart do divide in that period, this volume increase would correspond to 6.6 cell divisions, which is at least one division of each cell every 24 h. However, from literature (4, 23) and data obtained from quantitative reconstructions (21), we know that myocardial cells differ in cell-cycle duration within hearts and between hearts of different stages. The increase in volume may not only be explained by mitoses, but other mechanisms, such as cellular growth, cell migration, and transdifferentiation (recruitment) have to be taken into account as well. Therefore, an accurate description of the developing heart will benefit from data obtained by quantitative reconstructions (21). As illustrated, the current protocol can also be used for the 3D reconstruction of the expression patterns of heart-specific genes and transcription factors determined by qualitative (15) and quantitative (19) ISHs.

The correlation of gene expression data and quantitative morphological changes, both in their proper morphological context, will then serve to gain insight in the functional genomics of heart development.

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