Growth of the developing heart
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Citation for published version (APA):
van den Berg, G. (2011). Growth of the developing heart

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Chapter 3

3D Measurement and Visualisation of Morphogenesis: Applied to Cardiac Embryology


Abstract

Volume growth and proliferation are key processes in heart morphogenesis, yet their regionalization during development of the heart has been described only anecdotally. To study the contribution of cardiomyocyte proliferation to heart development, a quantitative reconstruction method was designed, allowing the local mapping of this morphogenetic process. First, a morphological surface reconstruction is made of the heart, using sections stained specifically for cardiomyocytes. Then, by a comprehensive series of image processing steps, local three-dimensional information of proliferation is obtained. These local quantitative data are then mapped onto the morphological surface reconstruction, resulting in a reconstruction which not only provides morphological information (qualitative), but also displays local information on proliferation rate (quantitative). The resulting 3D quantitative reconstructions revealed novel observations regarding the morphogenesis of the heart.
**Introduction**

The imaging of development has been described as ‘especially challenging because embryonic growth, tissue translocation and deformation result in a shifting terrain with moving landmarks that confound the analysis’ [1]. This is certainly true for the embryonic heart. The murine heart grows in width from 250 µm at embryonic day (E) 8.5 to 1.5 mm at E14.5 [2], which is an increase in volume from 2.5 nl to 1.3 µl in a few days. Despite this 500-fold increase in volume, the small size of the heart is still limiting the application of most molecular imaging techniques [3]. Because of the required level of detail and the limited penetration of staining agents in whole mount staining procedures, the use of serially sectioned biological material and 3D computer reconstructions is unavoidable [3,4]. Even though the time-dimension is lost in fixed specimens, the preservation of the true 3D geometry makes sectioning techniques extremely powerful for the study of embryonic development [1].

The rapid and intricate changes in cardiac morphology during embryogenesis are due to morphogenetic processes such as cell division and cell growth. Although a multitude of studies on cardiac cell proliferation exist, these studies present data outside a morphological context, or in a context which is hard to comprehend (for review see Soufan et al. [5]). Therefore, a 3D reconstruction protocol was developed to visualize and study the contribution of cell proliferation to cardiogenesis. This protocol is broken down into a qualitative (i.e. morphology) and quantitative (i.e. cell proliferation) method. The qualitative method identifies myocardium (i.e. cardiac muscle cells), resulting in a surface reconstruction. The quantitative method relies on a specific staining method to identify individual (proliferating) myocardial nuclei. The number of proliferating and non-proliferating nuclei is then systematically measured, providing local 3D information on cell proliferation rate. Mapping of these local data onto the morphological surface reconstruction results in a reconstruction that not only conveys morphological information, but is supplemented with quantitative morphogenetic data.
Methods

The 3D quantitative reconstruction protocol is broken down into two parts: a qualitative (morphological) reconstruction method and a 3D quantitative measurement method. Alignment, reconstruction and subsequent visualization of the images were done using the data analysis and geometry reconstruction program Amira (version 3.1; TGS Template Graphics Software, www.tgs.com). Image processing was done using Image-Pro Plus (version 5.0.2.9; Media Cybernetics, www.mediacy.com). In the current paper, the procedure is illustrated on a Hamburger and Hamilton stage 12 chicken embryo. The 3D quantitative reconstruction procedure was successfully applied to a time-series of embryos (n=10) ranging from stage 10 to 12 [5].

Histology

Sectioning

Detailed histology protocols have been published previously [6]. In short, the embryos were isolated, fixed, embedded in paraplast and cut into 7 µm thick serial sections which were mounted on 3-aminopropyltriethoxy-silane–coated slides. The chicken eggs were injected (in the yolk) with 100 µl of a 10 mg/ml solution of BrdU (5-Bromo-2'-deoxyUrIdine) in physiological salt solution (0.75 mOsmol/l). The embryos were thus exposed to BrdU for 4 hours prior to isolation.

Staining

The sections were deparafinized, rehydrated in a graded alcohol series and epitope retrieval was achieved by immersing the sections in 0.5 M HCl for 5 minutes, followed by a washing step of 3 x 5 minutes in PBS. The sections were then incubated overnight with a mixture of a mouse monoclonal antibody against BrdU (Becton Dickinson) diluted 1:100 in PBS with 0.05% Tween-20 (PBST) and a rabbit monoclonal antibody against cTnI (HyTest Ltd., Turku, Finland) diluted 1:250 in PBST. The sections were washed 3 x 5 minutes in PBS and incubated for 3 hrs in a mixture consisting of two labelled secondary antibodies and a nuclear stain. The secondary antibodies were goat-anti-mouse antibody coupled to Alexa 568 and goat-anti-rabbit antibody coupled to Alexa 647 (Molecular probes), both diluted 1:100 in PBST. The nuclear stain was Sytox Green (Molecular Probes), diluted 1:30,000 in PBST. All incubation steps using fluorochromes were shielded from light to prevent photo-bleaching. After incubation, the sections were washed in PBS for 3 x 5 minutes and mounted with Vectashield (Vector Laboratories Inc).
Confocal microscopy and image registration

The triple-stained sections were scanned at 10x magnification using a confocal scanning laser microscope (CSLM, MRC1024, Bio-Rad Microscopy Division, Helmstead, United Kingdom). To avoid spill-over of the fluorochromes, the sections were scanned sequentially using the specific laser lines and detection bands corresponding to the three individual fluorescent dyes. To reduce noise, the images were captured using the Kalman setting (4x). The Bio-Rad native .pic files produced by the CSLM contain a 8-bit greyscale image for each channel, i.e. the fluorescence of Sytox Green, Alexa 568 and Alexa 647, which correspond to the nuclei of all cells, nuclei of BrdU positive cells and myocardium, respectively. The images count 1024 pixels in x- and y-direction, each measuring 1.22µm.

The three 8-bit greyscale layers contained in the .pic files, were split into separate .tiff files to facilitate further image-processing. Thus, for every embryo three stacks of greyscale images are produced; myocardium (blue stack), BrdU positive nuclei (red stack) and all nuclei (green stack). The stacks were colour-coded for convenience. The images of the blue stack, displaying the myocardium, were registered using the align-module of Amira. Amira uses an iterative optimization algorithm based on grey-value correlation. Images failing to register automatically were manually aligned. The align-module only applies affine transformations, i.e. rotation and translation operations. After complete registration of the blue stack, the same translation and rotation parameters were applied to the red and green stack.

Morphological reconstruction method

The qualitative reconstruction method described below is a prerequisite for the quantitative reconstructions, but can also be used to obtain a morphological developmental series containing volume growth information [2].
**Tissue separation**

The surfaces used for the morphological reconstruction are based on the expression of cardiac Troponin-I [7] (cTnl), a specific protein expressed in heart muscle cells (myocardium) [8]. Separation of the myocardium from background was achieved through a custom-written thresholding macro. Comparison of the computer macro with human operators revealed that the macro performed sufficiently precise to be of practical use in the reconstruction procedure.

**3D Noise removal and visualization.**

The resulting stack of binary images was converted into a label-set, which is an Amira native file. Using Amira, information between the sections (z-direction) was interpolated to obtain isotropic voxels. The label-set was further smoothed and, after removal of histological artefacts (mainly gaps), converted into a 3D mesh of polygons (surface) which were visualized in 3D using a SurfaceView module (Fig. 1A).

**3D measurement procedure**

The procedures used in the qualitative reconstruction method can be used to obtain and visualize binary images of individual nuclei. However, such a direct display of all nuclei cannot be readily interpreted in terms of cell proliferation rate (cf. Fig. 1B). To obtain and convey such continuous information, the quantitative reconstruction method was developed. By means of a measurement of the number of nuclear profiles, local quantitative data were obtained. These can be mapped onto the surface obtained by the above morphological method thereby facilitating visualization and interpretation of cardiomyocyte proliferation.

**Profile identification**

The thresholded and binarized myocardium (blue) stack was used to mask the red and green stacks containing the nuclear profiles using a binary AND operation. This effectively removes all non-myocardial nuclei from the images in the red- and green stack while original pixel values in the nuclei within the myocardium are preserved. To quantify the number of nuclear profiles within the masked images, the profiles were identified and reduced to binary information in the form of an individual pixel (the centroid) per profile. The cell identification procedure was compared with human operator cell counting which showed that the automatic counting of nuclear profiles was sufficiently precise.
Local 3D Measurement.
The three binary stacks of processed images are merged into one stack of 8-bit images. The resulting stack of images thus contains combined binary information on the area of myocardium, the centroids of BrdU positive nuclear profiles and the centroids of all nuclear profiles. To convey the information in this stack in an interpretable way and to derive quantitative data, a 3D measurement procedure was implemented. A macro in Image-Pro Plus loads the entire stack of fused images into an x-y-z matrix (x and y equals the total image width and height, z equals the number of images). Subsequently this matrix is diced into cubes of 21x21x21 µm$^3$ of tissue volume spanning 3 sections. From the pixel value histogram in each of these measurement cubes, the total number of nuclear profiles ($N$) and the number of BrdU positive nuclear profiles ($N_p$) are derived. Using these values, the BrdU labelling index ($LI = N_p / N$) in each cube was calculated. The LI is encoded as an 8-bits integer number and exported to a new image resulting in a stack of images containing labelling index information (Fig. 1C).

3D visualization of cellular data
Amira has the functionality to project a block of 8-bits information (Fig. 1C) onto a surface (Fig. 1A). By projecting the 8-bits proliferation rate data onto the original myocardium surfaces (Fig. 1A) coupled to a colour-map, we obtained 3D quantitative reconstructions (Fig. 2A) in which the morphology of the heart -as well as- quantitative information on proliferation is presented.

Results
Noise removal in local 3D Measurements
The initial quantitative reconstructions obtained by the above-described method contained a lot of noise (Fig. 2A), due to the low number of profiles being counted in each measurement cube (i.e. 21x21x21 µm$^3$). These low counts result in discontinuities in the labelling index, especially at the periphery of the heart. To reduce the noise it was decided to increase the sample size, by measuring in a larger volume and to project the calculated data into the centre cube of the sample volume. In figure 2A, the measurement and projection cube were the same. The size of the measurement cube was step-wise increased by including 1, 2, 3, or 4 neighbouring cubes in x-, y-, and z- direction leading to sample volumes of $3^1$ up to $9^3$ cubes. The quantitative reconstructions of the labelling index data resulting from the inclusion of 1, 2 and 3 neighbours are shown in figure 2B, C and D, respectively.
An adverse effect of sampling in a larger volume is the loss of detailed information
and the inclusion of structures across a lumen. Therefore, it was required to select the optimal sampling size. Because of the wide range of labelling indices in the heart, a precision for the LI of 0.5 ± 0.075 was deemed sufficient. From the normal approximation of the binomial distribution, it can be calculated that such a precision can be reached with 95% confidence with 171 or more profiles per sample. The cumulative distribution plots of the number of profiles per sampling volume in the systematic series of quantitative reconstructions (Fig. 3) showed that 88% of the sampling cubes including 2 neighbours, dubbed S2, contain more than 171 profiles. Thus, in only 12% of the S2 quantitative reconstruction (Fig. 2C), the required quantitative precision would not be reached. The size of the S2 sampling cube (105³ µm³) was also deemed acceptable biologically because no smaller structures can be distinguished given the resolution of the morphological path of the reconstruction process. Thus, by measuring in a cube of 105³ µm³ and by mapping the quantitative information onto the 21³ µm³ centre cube of the sampling volume, the noise in the quantitative reconstructions could be reduced, without reducing the effective spatial resolution.

Figure 2: The 3D quantitative reconstructions using different sampling strategies in the local measurement step. In all reconstructions the projection cube (see text) is 21³µm³. The figure shows reconstructions in which the measurement cube is the same size as the projection cube (A; S0), in which the measurement cube includes 1 neighbouring cube in the x-, y- and z-direction (B; S1; 3³ cubes; 63³µm³), 2 layers of neighbours (C; S2; 5³ cubes; 105³µm³), and 3 layers of neighbours (C; S3; 7³ cubes; 147³µm³). The colour bar indicates the corresponding proliferation rate. Note that by increasing the size of the measurement cube, noise is reduced and the real spatial distribution of the proliferation pattern is recovered.
Figure 3: Cumulative frequency plot of the number of nuclear profiles counted in measurement cubes of increasing size. The measured volume for each of the traces is given as ‘times projection cube’ (see text and Fig. 2). This cumulative plot was used to determine the percentage of cubes containing more than 171 profiles (dotted line), which is the number of profiles required to estimate a labelling index of 0.5 with a 95% confidence interval of 0.075. In the reconstructions based on measurement cubes of 1, 3, and 5 cubes, the percentage of the local measurements that did not fulfil this precision criterion are 100%, 95%, and 12%, respectively. Therefore, in the reconstruction based on the 5 cubes, 88% of the labelling index information reached the required precision.

Biological Implementation

The current protocol was applied to gain insight into cell proliferation of the embryonic chicken heart. A timed series (n=10) of multiple reconstructions of cell division was made. The local changes in proliferation displayed a clear spatiotemporal pattern, yielding novel insights into the process of cardiac chamber formation [5]. For example, at stage 12 of chicken development (Fig. 4), regions of low and high proliferation are clearly identifiable. At this embryonic stage, the linear heart tube is undergoing rightward looping, attaining the classical c-shape. Two fast proliferating regions can be identified next to the low dividing parts of the tube: at the caudal side, at which the blood enters the heart (i.e. the inflow tract), and at the site where the primitive ventricle starts to balloon out. The latter observation supports the notion that the primitive ventricle balloons out due to an increase in cell-number [9-11]. The inflow tract is composed of rapidly dividing cells, which is a novel observation and fits with the theory that in this region newly formed mesenchyme differentiates into myocardium [5].
Discussion

3D reconstructions have recently shown significant potential in unravelling key aspects of morphological changes during cardiac development [2,12-16]. Regarding cellular proliferation in the early chicken heart, the pioneering studies of Sissman and Stalsberg in the 1960s have provided a wealth of meticulous observations on cellular proliferation [9,10]. These and other data, however, can only fully be exploited if incorporated into an approachable 3D context. It can even be argued, that the divergent opinions that exist regarding the cellular mechanisms underlying formation of the cardiac chambers take their origin from misinterpretations of the complex 3D architecture of the developing heart. To remedy this situation, a comprehensive 3D quantitative reconstruction protocol was developed and applied to early chicken cardiac development [5]. The protocol contains two automated procedures, one to identify myocardium on the basis of cardiac troponin I expression, the other to identify individual nuclei and reduce them to centroids. Both procedures were compared with human operators and showed that they were sufficiently precise (to be published).

The observed cell proliferation rate is dependent on the identification of myocardial nuclei. Fibroblasts have not yet entered the embryonic heart at the stages studied and the cardiomyocytes are still mononuclear [17], as the chicken cardiomyocytes become multinuclear only after hatching [18]. We have previously shown that ignoring the size difference between proliferating and quiescent cells does not significantly affect the estimation of proliferation rate [19]. The thymidine analogue BrdU is incorporated into dividing cells in the S-phase and can thus be used as a marker for proliferation. The labelling index, defined as fraction of labelled nuclei, is linearly related to the BrdU exposure time and reciprocal with the cell cycle length [20]. Therefore, with constant exposure time and S-phase length, the labelling index -with the current measurement protocol- can be regarded as a direct estimate of proliferation rate.

The current 3D quantitative reconstruction protocol can easily be adapted for the study of other morphogenetic processes (e.g. apoptosis) and can be applied to other organisms [19].

Acknowledgement

The authors were supported by a grant from the Netherlands Heart Foundation (M96.002). They wish to thank Piet de Boer and Jaco Hagoort for technical support.
Reference List


