Morphology, growth and patterning of the developing heart: methods and applications

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Summary
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Congenital cardiac malformations are among the leading birth defects in human live births, affecting 1 in 100 children. This high incidence of structural heart defects highlights the complexity of cardiac development and, indeed, heart development was shown to depend on an interplay of a large number of genes at different stages of development and in different compartments of the heart. In mouse, heart development takes place at a terrific pace. Developing, while functioning, in only a few days from a linear primitive tube to a complex four chambered heart. To enable this transformation, cells are added to the heart at both poles. Moreover, cells in the heart do not necessarily remain in the same compartment: cells from the outflow tract move into the right ventricle and cells from the atrioventricular canal into the left ventricle. Local differences in proliferation rate are another important parameter in heart morphogenesis.

To comprehend the processes involved in cardiac morphogenesis we need tools to understand morphology, to measure and visualize morphogenetic parameters and to interpret associations in gene expression patterns. Enormous amounts of in situ gene expression data are available in literature, but exploration of this wealth of information is hampered by the fact that each paper reports on a limited number of genes, studied in various developmental stages and species. To remedy this situation, several initiatives bring these data together in spatio-temporal gene expression atlases. In Chapter 1, eleven atlases, describing developing vertebrates and covering at least 100 genes, were reviewed. This review focused on: (1) the used anatomical framework, (2) the handling of input data and (3) the retrieval of information. The aim was to provide insights into the possibilities of the atlases, as well as to describe what more than a decade of developmental gene expression atlases can teach us about the requirements of the design of the ‘ideal atlas’. Most ingredients needed to develop the ideal atlas were already applied to some extent in at least one of the discussed atlases. The review concluded that the ideal atlas should be based on a spatial framework, i.e. a timed series of 3D reference models, which is anatomically annotated using an ontology with sufficient resolution, both for relations as well as for anatomical terms.

The development of a program for TRacing the Anatomical Context of Tissue Sections (TRACTS) was based on such a spatial framework. The aim of this program which fits 2D sections into 3D reference reconstructions and thus enables the retrieval of their correct location and orientation, was twofold. Firstly, placing sections in a cardiac reference model helps in the exact annotation of the cardiac compartments present in the section which will be of help to the non-morphological scientist in need of a histological confirmation of a gene expression profile. Moreover, much of the disagreement among embryologists may merely be based on miscommunication, because it is far from easy to form a mental image of a dynamically changing three-dimensional structure, even for experts. Secondly, TRACTS can be used to collect the gene expression patterns visible in the fitted sections within one common spatial framework. In Chapter 2 we show that a basic version of the program, using a primarily brute force pixel-based approach, already gave promising results. The performance of this basic program could be substantially improved when the program was extended with the use of some relatively simple image features. The initial performance of TRACTS, i.e. how well TRACTS fitted a section, was judged by experts in morphology. In this version of TRACTS, the reference model was reconstructed from histological sections stained with myocardium-specific markers.

In the final version of TRACTS, the spatial framework was improved by using a reconstruction based on episcopic images which are not subject to deformations due to sectioning and stretching. Now the performance of TRACTS was compared to that of the five experts in morphology
(Chapter 3). The program placed sections correctly, robustly and as precisely as the best of the fits achieved by the five experts. In the same chapter is described how 3D objects can be embedded into a pdf document. Without this, dissemination of 3D data is severely hampered by the 2D medium of print publication. Interpretation of the results of anatomical and embryological studies relies heavily on proper visualization of complex morphogenetic processes and patterns of gene expression in a three-dimensional (3D) context. Many insights gained from studying the 3D object are very hard to convey using only 2D images and are consequently lost or cannot be verified independently. We have developed a protocol that describes, step by step, how 3D objects can be embedded into a pdf document. This allows interaction with the 3D structure on one’s own computer screen which is ideal in the understanding of the intricate morphology of developing embryos. Both the use of TRACTS and the inclusion of 3D objects in pdf documents can help in the interpretation of 2D and 3D data, and will thus optimize communication on morphological issues in developmental biology.

The quantification of morphological parameters and their changes within a region of interest is a key concern in embryological studies. In Chapter 4, we describe a tool to measure the distance between two points which was used to measure the speed at which cells migrate from the growth center in the caudal pericardial wall into the non-proliferating primary heart tube. These distance measurements have to follow a track through the tissue when measuring in sheet-like or contorted organs like the developing heart. Three existing neighborhood estimators were compared; two of Verwer and one of Kiryati, all originally designed to compute chamfer distances in data sets with isotropic, cubic voxels. The estimators were, therefore, adjusted to handle non-isotropic data sets. Moreover, the shortest path along a user-defined track within a given tissue was calculated. The measurement of known distances, through a simplified model of an early heart tube with anisotropic voxels, was used to decide which of the three estimators should be implemented. The observed Root Mean Square (RMS) errors were similar to the ones reported in literature in the unrestrained isotropic case. The isotropy-adjusted Verwer estimator measuring in a 5³ neighborhood performed best by far with the lowest mean and RMS errors. Application of this method was used to show that cells from the growth center migrate into the primary heart tube at a speed of 70 µm per hour.

Much of our current knowledge on cardiac development is derived from the mouse model, permitting molecular analyses along with genetic lineage tracing. Important as these analyses are, they cannot fully be exploited when not supplemented with a clear insight of the growth of the embryonic heart, which is currently lacking. In Chapter 5, a comprehensive series of interactive 3D reconstructions is presented, showing the mouse heart development throughout the complete gestational period, each supplemented by the pattern of proliferation. We show that the splanchnic mesoderm is highly proliferative and that upon recruitment to the cardiac lineage the proliferation rate drops, as in human and chicken. These observations were done using morphological and quantitative 3D reconstructions. The latter are based on BrdU-labelling experiments. BrdU is a thymidine analogue, which is incorporated during DNA synthesis, in the S-phase, of the cell cycle. Proliferation rate locally increased at the sites of chamber formation, generating heterogeneous patterns of proliferation. Further quantitative analyses showed a gradual decrease in proliferation rate of the ventricular walls with progression of development, and a base-to-top decline in proliferation activity in the trabecules. Our data offer clear insights into the growth and morphogenesis of the mouse heart and provide a firm basis for future mechanistic studies.
Although the BrdU-labelling indices are related to the length of the cell cycle, a direct interpretation as such is hampered by the relative length of the S-phase compared to the total cell cycle length. Therefore, **Chapter 6** presents a method and a dedicated computer program to measure the actual local cell cycle lengths. This method relies on the labelling, for two different exposure times, with the two thymidine analogues IdU and CldU, in which the exposure times partly overlap. The observed difference in labelling indices, together with the known difference in exposure time, enables calculation of the cell cycle length. This method was applied on early chicken heart development and showed an extremely heterogeneous pattern of cell cycle lengths, with lengths ranging from less than 8 hours in the growth center in the caudal pericardial wall, less than 16 hours in the forming primitive ventricle, to days in the primary myocardium. The resulting quantitative 3D reconstructions of cell cycle lengths are the first to show this information based on labelling of 1 specimen.

As was described in chapter 1, anatomical annotation is often controversial because of anatomical nomenclature and definition of borders. These controversies can be, at least partially, solved by using annotated reference models. Since the meaning of terms used in anatomically annotated reference models will be clarified by their 3D spatial depiction in the models, it will be evident from the reference models what the defined borders of named structures are. This will remove most of the ambiguity in the terms used in anatomical annotations. A complicating aspect of using anatomical annotation is that gene expression patterns do not necessarily respect the borders of anatomical structures.

To address the latter problem it was proposed in chapter 1, to combine the anatomical annotation of a spatial framework with an annotation based on the expression profiles of a limited set of genes with known expression domains. To this end we spatially mapped, in **Chapter 7**, the expression patterns of 12 genes onto a previously published mouse reference heart of embryonic day 11.5. Hierarchical clustering was used to identify regions that have similar expression profiles. To restrict the number of branches of the dendrogram, we developed a dynamic pruning algorithm that does not require an arbitrary cut-off value. This algorithm performs a top-down pruning of the cluster tree based on the mean values of each gene in the profile per cluster and stops when the explained variance in the tree no longer increases significantly. This approach resulted in 18 continuous domains, each with its own unique expression profile. Most of these domains represented classical anatomical compartments, but we also observed novel domains that cannot be distinguished by any anatomical landmark.

The presented methods are tools to define and communicate on the anatomy and morphometrics of the developing heart. Application of the described methods provided new findings and biological insights into the morphogenesis of the heart. Our tools will enable the integration of quantitative morphological and gene expression data within a single spatial framework, which in turn, will allow a system biological approach of cardiac development.