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AUTOMATIC SEGMENTATION OF OPTICAL DENSITY IMAGES

Jaco Hagoort, Karim Salam, Jan M. Ruijter

Department of Anatomy and Embryology, Academic Medical Centre,
Meibergdreef 15, 1105 AZ Amsterdam, the Netherlands.
(e-mail: j.hagoort@amc.uva.nl)

ABSTRACT

Measurement of optical densities in different parts of an organ or tissue requires an objective segmentation of these different morphological structures in images of histochemically stained sections. However, in biological tissues, the relevant morphological structures often contain a wide range of pixel values which hampers the use of segmentation procedures based on thresholds, on region-growing or on a-priory knowledge of the shape of the segments. Segmentation based on multivariate statistics requires customised programs or dedicated hardware. Therefore, an automated segmentation procedure based on statistical criteria has been developed that could be used in the context of a readily available image processing package. The procedure is based on minimising the pixel value variation within each segment while maintaining the spatial continuity of the different segments in the image. In this procedure the original image is thresholded to make it binary and then subjected to binary operations (erosion and dilatation) to correct the spatial noise. The original image is then masked with the resulting binary image and its inverted complement. Of both partial images the variation in pixel values is measured and the pooled variation is calculated. The relative decrease of this pooled variation compared to the variation in the original image is used to decide whether or not the segmentation of the original image is considered relevant. If so, the image is split and the procedure is applied recursively on both resulting images until the reduction in variation no longer justifies a further segmentation. Reproducibility of the developed segmentation procedure was tested by applying it to pairs of normal and inverted images and to images with a compressed pixel value histogram. The proposed segmentation algorithm can be used for the segmentation of a known organ or tissue into functional zones, using a combination of fast image processing functions and statistical decisions.

Keywords: segmentation, threshold, binary operations, image analysis, statistics.

INTRODUCTION

Histochemistry, immunocytochemistry and in situ hybridisation are widely used in microscopic morphological research to show the presence of substances like enzymes, proteins, hormones and mRNA's in sections of fixed biological tissues. Quantitative

densitometric analysis of the products of these histochemical staining procedures plays an increasingly important role in the study of gene expression and functional zonation of tissues and organs (Jonker et al. 1997; Moorman et al. 1999). The aim of such a biological study is to distinguish functional zones within an organ, based on the optical density of a specific staining product. The shape of the structures, the differences in density and those of the density gradient between zones are unknown and may differ depending on the experimental or pathological condition of the tissue. This uncertainty hinders the use of knowledge-based segmentation procedures (Bartels and Thompson, 1994). Also segmentation based on tissue architecture like the spacing of cells (Geuzebroek et al. 1999) can not be used because most histochemical staining methods do not allow the segmentation of a clear marker for each cell. However, segmentation is a prerequisite for the extraction of useful densitometric information from optical density images of biological specimens.

The segmentation of images of biological specimens is hampered by the high variability of pixel values within morphologically continuous regions in the tissue. This pixel value variance, for instance non-staining nuclei surrounded by staining cytoplasm, is a property of the tissue and not the result of Poisson noise or technical shortcomings and should, therefore, be taken into account in the segmentation procedure. Simple or multiple thresholds, based on the pixel value histogram (Gonzalez and Woods 1993; Russ 1994) will lead to very fragmented segments riddled with holes. User intervention to correct this, either by manual thresholding or editing, will lead to user bias, especially because the ultimate aim of the procedure is to measure optical density levels in the resulting segments. Region-growing algorithms require a set of criteria for exploration of the neighbourhood of the seed pixels (Gonzalez and Woods 1993; Bartels and Thompson 1994). The variability within the regions makes it impossible to set these criteria. Similarly, this high variability will prevent split-and-merge algorithms (Bartels and Thompson 1994; Manousakas et al. 1998) from finding areas of uniformity. Alternatively this multilevel segmentation can be approached by using multivariate statistics, like cluster analysis. This procedure uses all variables known in individual pixels to classify them in a set of mutually exclusive groups (Chatfield and Collins 1983); within a group the pixels are similar, while pixels from different groups are dissimilar. This approach has been used for segmentation of pixel values in a time series dynamic scintigraphies (Hannequin et al. 1990). The spatial context of pixels can be included in this approach by using pixels values from synthetic images, created through some type of spatial filtering, as extra variables (Bengtsson et al. 1994). These procedures are computationally bulky, and require customised software and dedicated hardware (Bengtsson et al. 1994). Therefore, we decided to try a poor man's approach: development of a statistical segmentation procedure that could be used in the environment of a readily available image processing software package (NIH 1997). The procedure combines the fast image processing functions of the NIH-Image package (thresholding, binary and mathematical operations and measurement) with a statistical decision algorithm. The resulting image segments are spatially continuous and the pixel value variation within these segments is maximally reduced. The method should result in reproducible segments for images with the same overall structure but different pixel value distributions. More exactly, the segmentation procedure should give identical results for inverted images and for images with a compressed pixel value histogram. Application of the procedure on serial sections of the same tissue should result in similar segments. The presented segmentation procedure is based on a recursive sequence of pixel level thresholding, morphological correction of the resulting binary images and a statistical decision on whether or not to continue segmenting the resulting partial images.

PROGRAM DESCRIPTION

All steps of the segmentation procedure will be illustrated with an image of a section of a rat heart of 13 embryonic days hybridised with a radioactive probe for SERCA2 mRNA (Moorman et al 1995; Fig. 1A; high pixel values are shown in black). The segmentation procedure was developed as a macro for the image analysis package NIH-Image (NIH 1997) and is written in the Pascal dialect of this package. However, the image processing functions used are available in all image analysis packages, allowing this segmentation procedure to be readily converted to other environments.

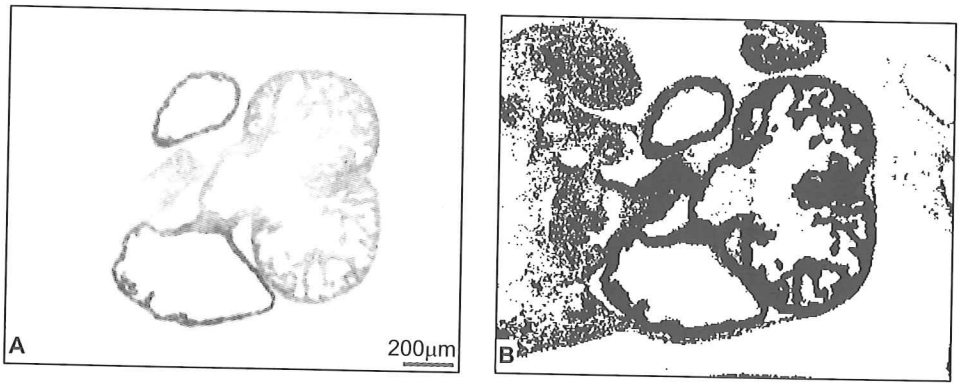

Fig. 1. ***A.*** *Optical density image of a section of a rat heart of 13 embryonic days of development hybridised with a radioactive probe for SERCA2 mRNA and processed for autoradiography.* ***B.*** *The same image after the application of a single threshold and binarisation.*

Recursion. The main part of the segmentation procedure (Fig. 2) is a recursive operation that consists of the thresholding and binarisation of the input image, the morphological correction of the binary image, the measurement of the pixel value variation in the resulting image parts and the statistical decision whether or not the splitting of the input image leads to a considerable decrease of the pixel value variation. If so, the input image is split and the procedure is repeated for both parts resulting in a dichotomous tree of image segments.

Thresholding. In the automatic threshold function of NIH-Image, the threshold is defined as the pixel value that separates the histogram of the product of frequency and pixel value into two equal parts, in effect using the pixel value as a weight factor in the histogram separation. To avoid this weight factor, a thresholding function based on just the frequency was incorporated in the procedure. This threshold was set at a point where the sum of frequencies of the low value pixels was twice the sum of that of the high value pixels. This was done to improve the resolution of the segmentation in the low frequency (= high optical density) part of the histogram, where in our experience the interesting structures are represented. After setting the threshold, the image is converted into a binary image (Fig. 1B). Copying and inverting this binary image results in two complementary binary images, each representing a phase of the input image.

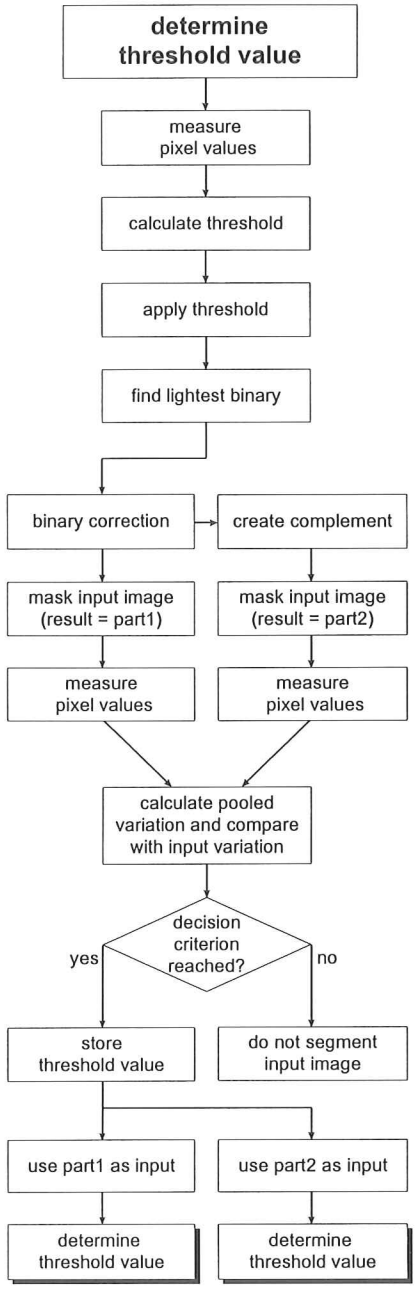

Fig. 2. Flow chart of the recursive part of the segmentation procedure.

Binary morphological correction. After thresholding and binarisation the resulting images have to be subjected to a series of binary erosion and dilation operations to correct for spatial noise (Fig. 3). Since the effect of erosion and dilation is dependent on the pixel distribution in the image, correcting both binary images would result in images that are no longer complementary. Therefore, we choose to start the iterative series of erosions and dilations that form the morphological correction, always with an erosion in the lightest binary image of the pair. The other binary image is then reconstructed as the complement of the corrected image.

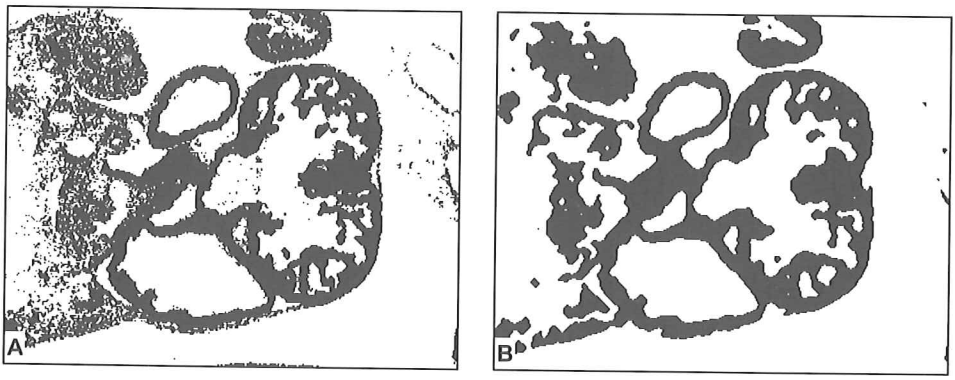

Fig. 3. A. Uncorrected binary image. B. Corrected binary image after an erosion - dilation sequence (4 iterations).

Masking of the original. Both complementary binary images are used to mask the original image by using a binary AND operation. This results in a pair of complementary images (Fig. 4). In each segment a range of pixel values is present but the mean pixel value of both segments is different (Fig. 5, recursion level 1).

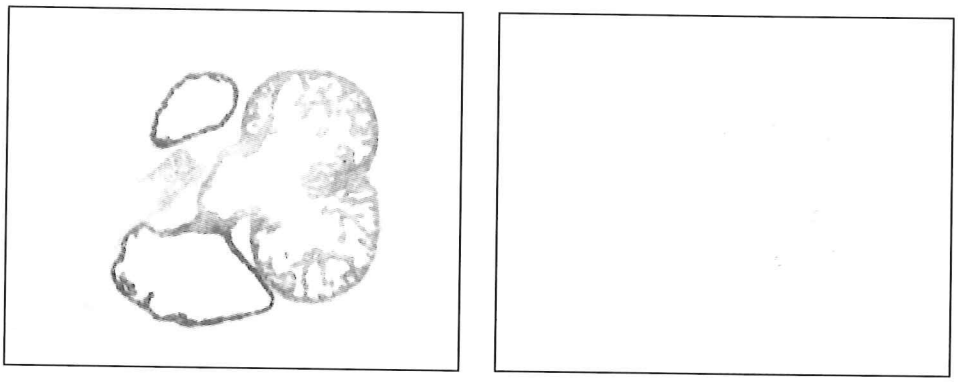

Fig. 4. Pair of complementary images resulting from one sequence of thresholding, binary corrections and masking of the original image.

Measurement and calculation of variation. In the input image and in each member of the resulting image pair, the standard deviation of the pixel values (excluding the white phase which represents the area occupied by the other segment of the image) is measured.

From this standard deviation, the pixel value variation in the image is calculated by squaring the standard deviation and multiplying by the number of non-white pixels minus 1.

Statistical decision. The variation of the two image parts is summed to calculate the pooled variation after segmentation. This pooled variation is then compared with the variation of the input image. The pooled variation will generally be lower then the input variation showing that the segmentation of the image reduces the overall variation. When the reduction of the variation exceeds a predetermined criterion, e.g. 30%, the segmentation of the original image is considered relevant and the segmentation procedure is repeated for both image segments thus leading to a dichotomous tree of image segments (Fig. 5). When not enough reduction of the variation is reached the input image is considered to be an endpoint in that branch of the image segmentation tree. When all image parts are marked as endpoints the recursive segmentation procedure stops.

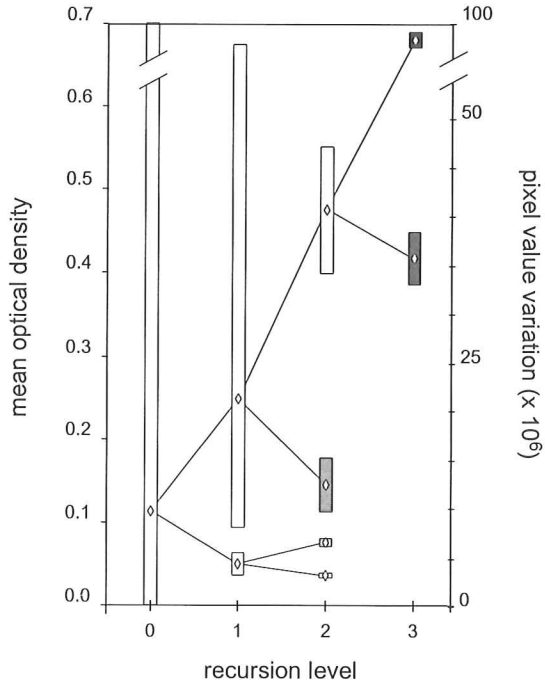

Fig. 5. Image segmentation tree. Mean optical density (diamonds) and pixel value variation (bars) in the dichotomous tree of image segments. The graph shows the measurement results after each recursion of the segmentation procedure. Recursion level 0 is the original complete image. The measured variation of each partial image is shown as a bar. The reduction of the pooled variation with each recursion level is clearly visible

Combining resulting segments. During the course of the segmentation procedure the thresholds that have lead to relevant splits are collected in memory. For the creation of a combined image with all segments, these thresholds are sorted and applied consecutively to the original image. After applying the same binary morphological corrections each segment is assigned an ordered pixel value. The resulting segmented image thus resembles the original in the relative intensity of pixel values (Fig. 6).

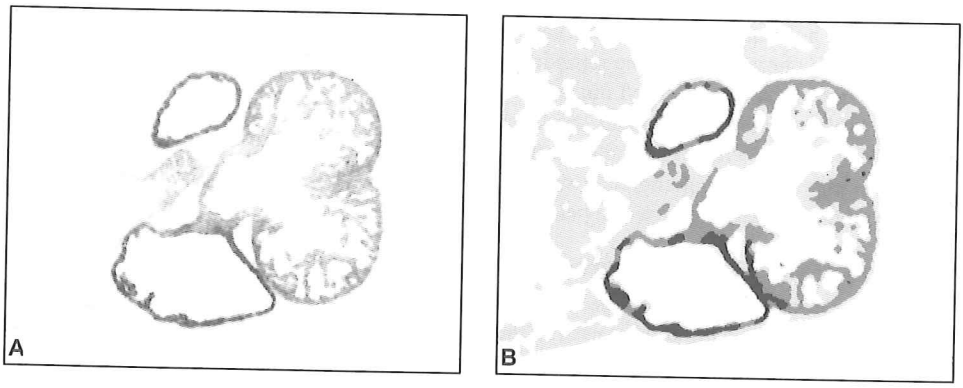

Fig. 6. ***A.*** *Input image.* ***B.*** *Segmented image with 5 segments. The decision criterion was set at 70. Intensity coding of the segments in panel* ***B*** *is the same as in Fig. 5.*

RESULTS AND DISCUSSION

Reproducibility

The presented segmentation procedure fulfils the requirements for reproducibility stated on the outset. When the procedure was used to process images with an inverted pixel value distribution, the resulting segmented images corresponded exactly (Fig. 7) with the segments of the original images (Fig. 6).

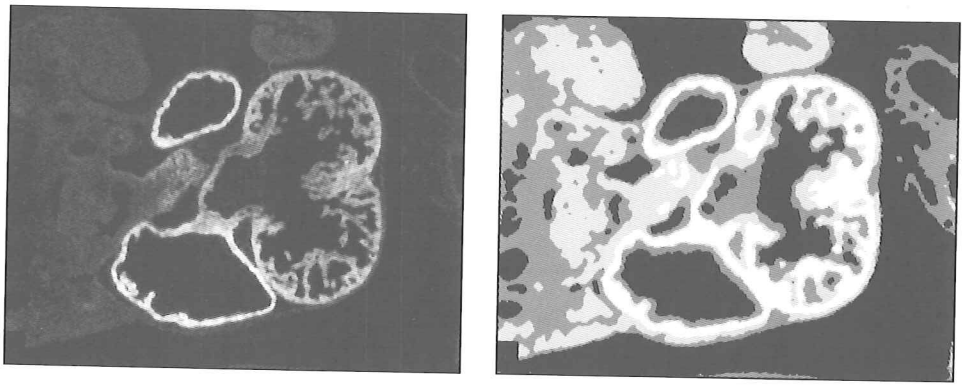

Fig. 7. A. Inverted input image. B. Segmented image. Decision criterion set at 70. The segmentation result in panel B is identical to that in Fig. 6B

Also shifting the pixel value distribution by compressing the histogram (thus simulating the effect of poor lighting conditions or increased deposit of staining product) did not affect the segmentation result. This reproducibility was achieved by carefully designing two of the key functions of the procedure: the thresholding and the binary morphological correction. The standard automatic thresholding function of NIH-Image proved unsuitable for

our purpose because it uses a pixel value weighted frequency distribution. Such a weighted threshold will always hamper the reproducibility of the segmentation procedure when applied to inverted or pixel value shifted images. When just the frequency distribution of pixel values is used to determine the threshold level, one guarantees that segmentation of those inverted and shifted images will give results identical to the original. However, a prerequisite for this reproducibility is the strict choice to start the binary morphological correction with an erosion operation on the lightest image of a pair of binary threshold results.

Effect of the statistical decision criterion

The third key function in the presented segmentation procedure is the statistical decision. The decision criterion is used in the recursive step of the procedure to decide whether or not each splitting of the input image is relevant. A decision criterion set at 70 means that a reduction in pixel value variation with 30% or more is required while a criterion set at 90 only requires a 10% reduction in variation. Since the latter requirement is more readily reached, more segments will result from a criterion of 90.

In a series of tests with optical density images of in situ hybridisations of heart and liver, the effect of this decision criterion on the number of generated segments was tested. As can be expected the number of resulting segments increases when the requirement for decreased variation is relaxed (Fig. 8). Although the determination of the decision criterion involves a subjective choice, the tests we did with a range of decision criteria shows that in all tissues one can find a limited range of criteria from which the same number of segments results. This indicates that in that range of criteria the segmentation result is not dependent on the image contents. The preferred choice for a decision criterion should therefore be in the middle of this range of decision criteria. Since a series of related images will probably have similar pixel value distributions, once a decision criterion is set, segmentation of the whole series will result in a similar number of segments in each image. Tests done with adjacent serial sections of the same tissue confirm this.

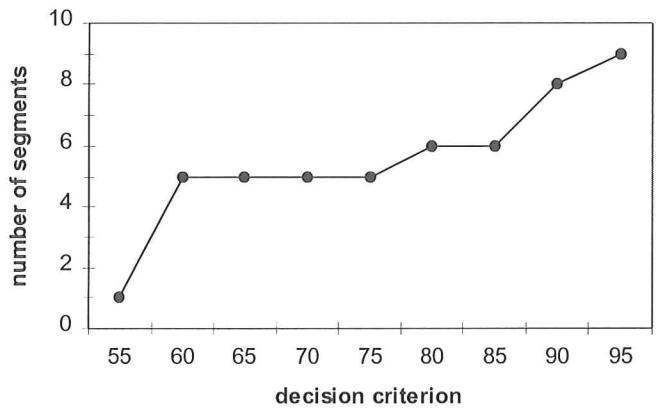

Fig. 8. Effect of the decision criterion on the number of recognised segments in the optical density image of the 13 embryonic day rat heart. In the range of decision criteria of 60 to 85 (requiring 15 to 40 percent reduction in pixel value variation to split an image into two parts) the number of segments remains constant.

The customary way to treat the reduction in pixel value variation is to calculate the variances of pixel values in stead of the variations. These variances can be compared with an F-test, assigning one degree of freedom to the reduction in variance (Snedecor and Cochran 1982). However, the variances are calculated over such a high number of pixels that this F-test will always give an extremely low p-value, necessitating an arbitrary choice of significance level to use this test result. Because of this, it was decided to base the statistical procedure on just comparing the variation of the input image and the pooled variation of the segmented parts.

CONCLUSION

The segmentation procedure described above is based on thresholding and morphological correction of segments, leading to spatially continuous areas with minimised internal pixel value variation. The procedure has been successfully applied to images of different biological tissues. Visual inspection of the results shows that the segmented areas closely resemble those seen by the user. However, the procedure works without human interaction and is therefore free of user bias. Comparison of the segmentation result with human performance in tracing is not a valid test for accuracy of the procedure: different users will place boundaries based on different implicit criteria. The presented method is detached from user subjectivity by a pre-defined thresholding setting, a fixed binary correction algorithm and adjustable statistical decision criteria. The latter only influences the number of segments, adding extra segments does not affect the boundaries of those already placed. This objectivity and the reproducibility of the procedure make it a valuable tool in the study of functional zonation in biological tissues and organs. This segmentation procedure can be used in the macro environment of a readily available image processing package (NIH 1997). Although it is written in the Pascal dialect of NIH-Image it can easily be converted to other image analysis packages that allow user-written inserts or macro programming.

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REFERENCES

Bartels PH, Thompson D. Scene segmentation. In: Marchevsky AM, Bartels PH eds. Image analysis: a primer for pathologists. New York : Raven Press, 1994: 57-77.

Bengtsson E, Nordin B, Pedersen F. MUSE - a new tool for interactive image analysis and segmentation based on multivariate analysis. Computer Methods Programs Biomedicine 1994; 42: 181-200.

Chatfield C, Collins AJ. Introduction to multivariate analysis. London: Chapman and Hall, 1994.

Geuzebroek J-M, Smeulders AWM, Cornelissen F, Geerts H. Segmentation of tissue architecture by distance graph matching. 1999; 35: 11-22.

Gonzalez RC, Woods RE. Digital image processing. Reading : Addison-Wessley, 1993.

Hannequin P, Liehn JC, Valeyre J. Cluster analysis for automatic image segmentation in

dynamic scintigraphies. Nuclear Medicine Comm 1990;11: 383-393.

Jonker A, de Boer PAJ, van den Hoff MJB, Lamers WH, Moorman AFM. Towards quantitative in situ hybridisation. J Histochemistry Cytochemistry 1997; 45: 413-423.

Manousakas IN, Undrill, PE Cameron GG, Redpath TW. Split-and-merge segmentation of magnetic resonance medical images: performance evaluation and extension to three dimensions. Computers Biomedical Research 1997; 31: 393-412.

Moorman AFM, Vermeulen JLM, Koban MU, Schwartz K, Lamers WH, Boheler KR. Patterns of expression of sarcoplasmic reticulum Ca^{2+} ATPase and phospholamban mRNAs during rat heart development. Circulation Research 1995: 76: 616-625.

Moorman AFM, De Boer PAJ, Hagoort J, Franco D Lamers WH. Radio-isotopic *in situ* hybridization on tissue sections: practical aspect and quantification. Developmental Biology Protocols 1999; in press.

NIH NIH-Image: image processing software package. Version 1.61. 1997. Available from: http://rsb.info.nih.gov/nih-image/

Russ JC. The image processing handbook. Boca Raton: CRC Press, 1994.

Snedecor GW, Cochran WG. Statistical Methods. Ames: Iowa State University Press, 1982.

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