ACTA STEREOL 1999; 18/3: 361-366
ORIGINAL SCIENTIFIC PAPER

DENSITOMETRIC AND STEREOLOGICAL DETERMINATION OF ZONAL GENE EXPRESSION IN LIVER.

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ABSTRACT

The expression of many hepatic genes depends on the position of the hepatocyte along the sinusoids connecting the terminal branches of the portal vein and the central vein. The expression pattern along this porto-central axis may differ strongly for different genes or under varying metabolic conditions. Hepatocyte gene expression can be visualised by in situ hybridisation on tissue sections. Previous tests have shown that the optical density (OD) of the resulting sections is proportional to the amount of gene product present in the tissue. To obtain a graphical representation of this expression pattern a measurement procedure based on a combination of densitometry and a stereological model was developed. In this model the liver lobulus is considered to be a convoluted cylindrical tube which is randomly oriented with respect to the plane of sectioning; different gene expression levels can then be pictured as concentric circles or ellipses of hepatocytes with increasing or decreasing OD. The cumulative area occupied by a class of hepatocytes with similar OD and all classes between this class and the centre of the lobulus, expressed as fraction of the total area, is equal to the square of the outer limit of this class, expressed as a fraction of the radius of the lobulus.

Applying this model to actual OD images of liver sections involves segmentation of the OD values in the image into a number of zones. This segmentation is carried out with an automatic procedure that removes spatial noise. The area of the resulting concentric zones and the mean OD of the same area, masked in the original image, are measured. The area occupied by the portal- and central veins is measured separately. The relative position of the outer border of each zone on the radius of the liver lobulus can then be calculated by taking the square root of the values in the relative cumulative area distribution. The resulting graphs show on the Y-axis the mean optical density per class and on the X-axis the position of this zone on the porto-central radius of the lobulus. The results obtained by this approach are reproducible and fitting the data to a model for the regulation of gene expression in the liver can be used to extract kinetic parameters.

KEYWORDS: densitometry, stereology, gene expression, liver, hepatocyte .

INTRODUCTION

In situ hybridisation with cRNA probes is used to visualise the localisation of gene expression in tissues. Recently it was shown that this technique can be used such a way that it not only gives qualitative but also quantitative information on gene expression in tissue sections (Jonker et al. 1997). The optical density measurements, integrated over the tissue area, are linearly related to the total amount of gene product present in the tissue. In many instances gene expression in organs is not homogeneously distributed over the tissue but restricted to specific cells. In these cases zonal pattern and gradients therein are observed.

The expression of genes, encoding for metabolic enzymes in the liver, follows such a zonal pattern. This pattern can be described in terms of the smallest architectural unit of the liver: the liver lobulus. Some enzymes are preferentially expressed by hepatocytes in the vicinity of the terminal branches of the portal vein (PV) (so-called periportal hepatocytes) whereas, other enzymes are restricted to the area around the terminal branch of the hepatic vein, the central vein (CV) of the liver lobulus (pericentral hepatocytes). An example of an enzyme with a strict pericentral expression pattern is glutamine synthetase (GS) that is found in a narrow rim of 2-3 cells thick around the central veins (Fig. 1A).

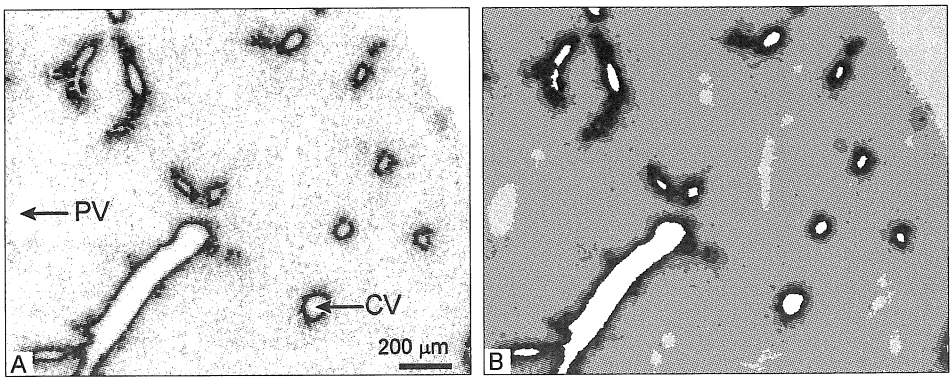

Fig. 1**A** Image of a mouse liver section of, in which glutamine synthetase mRNA is visualised with an in situ hybridisation procedure employing ^{35}S cRNA probe. **B**. The optical density image of 1A was segmented; apart from the areas occupied by the central and portal vein, 5 zones were recognised.

A method to obtain a graphical representation of the quantified expression of zonally expressed liver enzymes has been described (Lamers et al. 1997). This method is based on a stereological reconstruction of the density zones in an image of a hybridised liver section. The resulting graphical representation of differential gene expression as a function of its localisation in the tissue does not allow statistical comparisons between observations in order to detect differences between experimental conditions, i.e. hormonal induction. To overcome this limitation we extended the graphical method in two ways. First the segmentation of the images into zones of differential expression was automated to allow for an objective user independent analysis. Second the resulting graphs were used as input data for a model describing gene expression (Christoffels et al. submitted) in which kinetic parameters could be determined. This model is based on the hypothesis that zonal expression in hepatocytes is determined by their position; namely dependent on their localisation between the portal and central veins (porto-central axis). The purpose of the quantitative image analysis described here is to determine the relation between the level of gene expression, quantified as optical density, and the position of the hepatocytes along the porto-central axis.

STEREOLOGICAL MODEL

Through the combination of densitometry and area measurement the stereological model proposed here is used to reconstruct a biological process that can be described on a one dimensional scale from observations in two dimensional sections. Optical density measurements in lobular areas are translated into gene expression levels along the lobular radius.

Porto-central position. The stereological model used for the reconstruction of zonal gene expression in the liver is based on the assumption that the functional unit of the liver, the liver lobulus can be modelled as a convoluted cylindrical column surrounding the central vein with the terminal branches of the portal vein at the periphery of the cylinder (Fig. 2). The expression patterns of genes that increase or decrease along the radius of the lobulus, can be visualised in this model as concentric circles or ellipses with increasing or decreasing expression levels. Assuming a random orientation of the plane of sectioning with respect to axis of the lobulus, the relative cumulative area of each expression zone is proportionally related to the square of the distance of the outer boundary of the zone to the centre of the lobulus [eq.1].

$$\text{outer boundary zone} = \sqrt{\text{relative cumulative area}} \qquad \text{[Eq. 1]}$$

When the cumulative area is set to 100, Eq. 1 leads to a standardised lobulus with a radius of 10. The position of the expression zones along the porto-central axis can thus be calculated from the contribution of each zone to the cumulative area distribution.

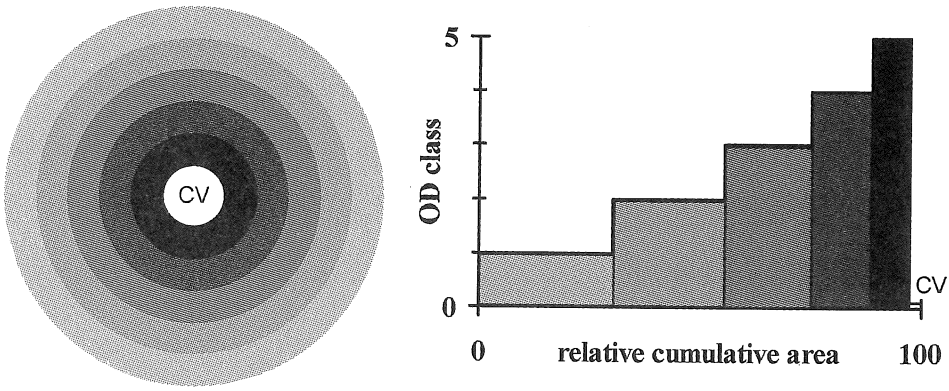

Figure 2. Schematic illustration of the stereological model. The liver lobulus is modelled as a cylindrical column with an increasing density gradient from the periphery to the centre and divided in five classes surrounding the central vein. The cumulative area distribution of the density zones is plotted against the optical density class.

Expression level. The mean optical density of the hepatocytes in each zone is proportionally related to the concentration of expression product in these cells and can therefore be used as a measure for the level of gene expression. In order to maintain the linear relation between expression levels measured as optical density and the actual amount of expression product present in the tissue the optical density should not be higher than 1, equivalent to a transmission of 10%.

Parameter estimation. The porto-central positions and associated optical densities thus obtained are used to fit a model for zonal gene expression based on receptor - ligand binding (Christoffels et al. submitted). In this model (Eq. 2) the expression level on a 0 - 1 scale (R) of a specific gene in the hepatocyte depends on the concentration of a regulatory transcription factor (S), the affinity (K) between this transcription factor and DNA and the degree of cooperativity (n) in the binding of the transcription factor to the DNA.

$$R = [S]^n / ([S]^n * K^n) \qquad \text{[Eq. 2]}$$

To fit this model, the activity of the transcription factor assumed to be directly related to a signal gradient along the porto-central radius.

DESCRIPTION OF PROCEDURES

Images are acquired from liver sections labelled with in situ hybridisation to visualise the expression of GS mRNA. Each acquired image is converted to an optical density image with the use of an empty image acquired at the same microscope settings (Jonker et al. 1997).

Image segmentation. The optical density image is subjected to an automated segmentation procedure (Hagoort et al. this issue). In short, this segmentation procedure consists of a recursive loop containing the thresholding of the input image, binary morphological corrections of the binary image, masking of the original image, measurement of

the grey value variation in the image parts and the statistical decision whether or not the splitting of the input image leads to a relevant decrease of the grey value variation. Applied to GS-hybridised liver sections this procedure results in a segmented image in which each segment represents a zone of differential gene expression (Fig. 1B). However, small, low density tissue components like cell nuclei and the lumen of sinusoids do not affect the segmentation result because the binary morphological correction removes such 'noise'. The segmentation result clearly shows the pattern of concentric density zones with increasing density from portal veins to central veins.

Identification of blood vessels. The central vein occupies an area at the centre of the standardised lobulus (Fig. 2B) and the measurement of this area is needed for correct calculations. The central and portal veins are interactively delineated on the original image and copied into the segmented image. The area occupied by portal veins is discarded.

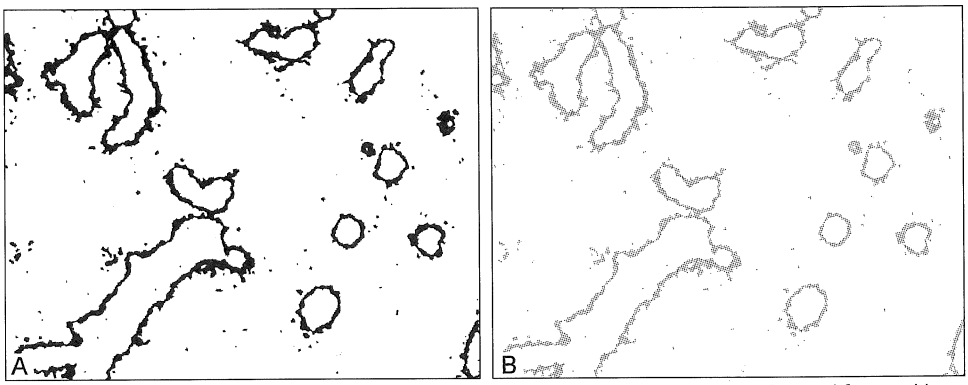

Figure 3. **A.** One zone of the segmented image (Fig. 1B) is isolated. **B.** This zone is used for masking the original image (binary AND). The resulting grey value image contains only the current zone and is used for measurement of area and mean optical density.

Measurement per zone. After segmentation the area and mean optical density of each concentric zone is measured (Fig. 3). To that end each zone is isolated from the segmented image and made binary (Fig. 3A). The original input image is masked with this binary image using a binary AND operation. In the resulting grey value image (Fig. 3B) the area and optical density of the current zone is measured excluding the white pixels. This procedure is repeated for every zone. When all zones in an image are measured a cumulative area distribution is constructed, starting with the central vein area and consecutively adding the areas from central to portal. The resulting total area is set to 100 (Fig. 4).

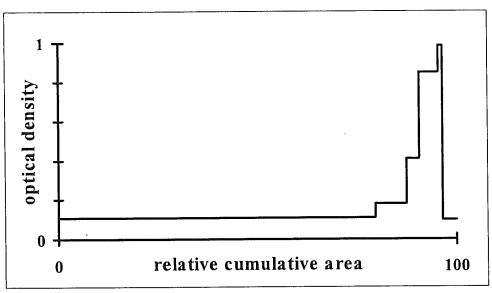

Figure 4. Relative cumulative area distribution of the image in Fig. 2. The Y-axis displays the mean optical density measured in the lobular zones. On the X-axis the contribution of each zones to the cumulative area distribution is plotted.

Calculation of the porto-central position. From the resulting relative cumulative area distribution the outer boundary of each lobular zone is calculated using the square root (Eq. 1) and this result is corrected for the radius of the central vein by setting this radius to 0 and proportionally recalculating all other boundaries (Fig. 5A). The resulting relative positions on the central portal axis are then converted into positions on the porto-central radius. Simultaneously the boundaries of the zones are used to calculate the middle of each zone. A scatter plot of latter value against the mean optical density of each zone results in a graphical visualisation of the density distribution over the porto-central axis of the liver lobulus (Fig. 5B).

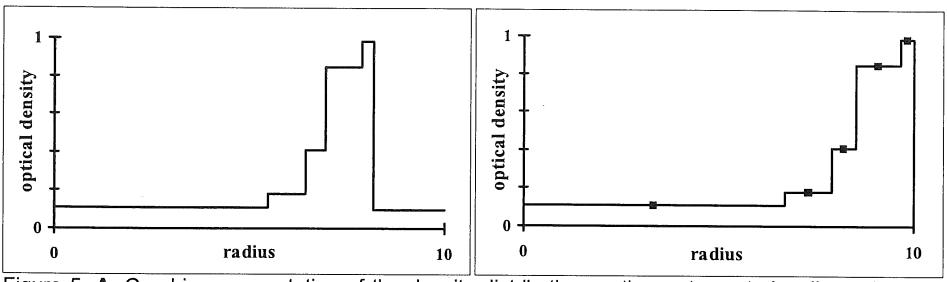

Figure 5. **A.** Graphic representation of the density distribution on the porto-central radius calculated from the measurements in the image of Fig. 1A. **B.** Density distribution of **A** corrected for the radius of the central vein. Symbols represent middles of zones. In both graphs the X-axis displays the result of optical density measurements.

Curve fitting. The results of all images of one experimental subject are pooled into one set of position and optical density data. The model for gene expression (Eq. 2) is fitted to this pooled data set. To this end the optical density values were normalised by setting the maximum value to 1. This is necessary because Eq. 2 results in values between 0 and 1. In order to correct for non-specific labelling, the normalised minimum optical density value was used as an offset. This resulted in Eq. 3 which was used in the curve fitting procedure (non-linear regression; SPSS).

$$\text{Normalised OD} = \text{offset} + (1-\text{offset}) \times [S]^n / ([S]^n \times K^n) \qquad \text{[Eq. 3]}$$

The signal concentration S was assumed to linearly increase from 0.1 to 0.3 over the whole length of the porto-central axis. The result of the curve fitting is given in Fig. 6.The line, calculated according to the model, closely fits the data points. The resulting kinetic parameters K and n can be used as explanatory parameters in a qualitative description of gene expression as well as for statistical comparisons between groups of observations in different experimental conditions.

DISCUSSION

The presented method generates a quantitative description of the relation between the gene expression level in hepatocytes and their position along the porto-central radius liver lobulus. The proposed stereological model takes into account the entire image and therefore avoids the bias that may be introduced by an analysis that requires the user to identify the portal and central ends of the gradient. The main requirement of the model is complete randomness of the direction of sectioning with respect to the axis of the hepatic lobulus. Since the lobulus is generally modelled as a convoluted tube, this requirement is fulfilled. Takahashi (1970) has shown that the hepatic vascular tree is characterised by regular interdigitation of the portal and central veins with uniform distance so that the implicit assumption of the model that is that the steepness of the gradient of gene expression along the porto-central radius proportional to the length of this radius, is also fulfilled.

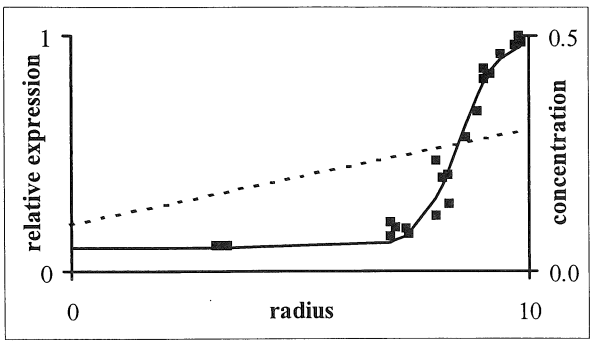

Figure 6. Graph showing the pooled data points measured in a series of 5 images of liver sections. The dotted line represents the assumed concentration gradient of the signal. The fitted line is based on the model regulation of liver gene expression described in the text (R^2 = 0.975). The estimated values for the parameters are: K = 0.27 ± 0.001 and n = 31.5 ± 2.6.

The method described here can only be used for gradients in gene expression that monotonously increase or decrease along the porto-central axis since the order in which the zones in the segmented image are measurement is solely dependent on their mean optical density. Additional image analysis functions have to be implemented to apply this method to gradients with a highest or lowest value somewhere along the radius of the lobulus (Lamers et al. 1997).

A kinetic model for gene expression (Eq. 2) is used for curve fitting with the data sets with positional and optical density information. The good fit of this model to the data set in the presented example has also been found for expression patterns of other enzymes with increasing as well as decreasing porto-central gradients (Christoffels et al. submitted). The use of this model complements the image analysis procedures since it offers the possibility to go beyond the realm of qualitative visualisation. The calculation of kinetic parameters enables a true quantitative comparison of gene expression between individuals, groups and experimental conditions.

REFERENCES

Christoffels VM, Sassi H, Ruijter JM, Moorman AFM, Grange T, Lamers WH. A mechanistic model for the emergence and maintenance of porto-central gradients in gene expression in the liver. (submitted)

Hagoort J, Salam K, Ruijter JM. Automatic segmentation of optical density images. (This issue)

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

Lamers WH, Geerts WJC Jonker A, Verbeek FJ Wagenaar GTM, Moorman AFM. Quantitative graphical description of porto-central gradients in hepatic gene expression by image analysis. Hepatology 1997;26:398-406

Takahashi T. Lobular structure of the human liver from the viewpoint of hepatic vascular architecture. Tohoku J Exp Med 1970;101:119-140