MORPHOMETRIC ASSESSMENTS FOR THE HEALTHY RAT HEPATOCYTES

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Summary
The purpose of this study is to perform an evaluation of representative size and shape parameters that characterise the hepatocytes in normal rat livers. A set of thirty digital images of rat hepatocytes in normal rat livers, randomly selected from a database, were evaluated. Image processing and analysis of digital images were performed with ImageJ and MRI Cell Image Analyzer (MRI-CIA) softwares. We found a set of representative size and shape parameters for quantification of hepatocytes in healthy rats. The central tendency and dispersion measure of the parameters were expressed by the mean value and standard deviation. The computerized geometric morphometric analysis was in agreement with the ultrastructural observations. Geometrical morphometric analysis of the normal rat livers structure is an efficient noninvasive prediction tool for analysis of rat histological images.

Keywords: image analysis, rat liver, hepatocyte ultrastructure, morphometry, shape, shape descriptor

Introduction
Over the last two decades, in the field of histology, different methods to analyze the structure of rat cells and tissues for the evaluation of health or the detection of the liver disease were performed (Ivanovska et al., 2010; Haidekker, 2011).

These methods and measurements proved sufficient for some studies (Ivanovska et al., 2010; Nailon, 2010), however they are less well suited for quantifying the rat hepatocyte ultrastructure (normal and pathological) concerning the cell shape, size and tissue hierarchical structure.

The modern computerized geometric morphometric methods have found wide application in the biological sciences and provide a better characterisation in describing the complexity of anatomical structures (Richtsmeier et al. 2002; Grizzi and Chiriva-Internati, 2005; Russ, 2007).

Computational tools for the analysis of cell shape allow to extract a quantitative and statistically valid understanding of the relationship between cell shape and experimental conditions (Pincus and Theriot, 2007; Nalakarn et al., 2007).

The analysis of complex anatomical systems requires many descriptors (Grizzi and Chiriva-Internati, 2005).

Shape descriptors have proven to be a useful tool for biological and medical image processing applications. Various shape descriptors exist in the literature for 2D and 3D images, mainly categorised into two groups: contour-based shape descriptors and region-based shape descriptors. Contour-based approaches are more popular than region-based approaches in biological and medical applications, but...
region-base methods are more robust as
they use all the shape information available
(Zhang and Lu, 2004; Martinez-Ortiz,
2010).

The shape descriptors depend on the
methodological and experimental
parameters involved, as: diversity of
subjects, image acquisition, type of image,
image quality, its processing, analysis
methods, including the algorithm and
specific calculation used (Russ, 2007; Talu,
2012). On the other hand, they are highly
efficient in measuring the cell hierarchical
structure and in revealing very small-scale
variations (Martinez-Ortiz, 2010).

In our study we have investigated
the normal rat hepatocytes using
computerized geometric morphometrics.

Material and methods
The experiments have been carried
out on male, adult Wistar rats, weighing
180-200 g. The group of rats (10 males),
received the standard diet and tap water ad
libitum.

The experiment lasted for 30 days;
in the end the animals were sacrificed, liver
samples were collected and properly
processed for structural and ultrastructural
investigations.

For ultrastructural investigations,
pieces of hepatic tissue harvested
immediately after killing the animal were
prefixed at room temperature, for 2 hours,
in a 2.7 % glutaraldehyde solution in
phosphate buffer 0.1 M, pH 7.2.

Samples were then washed in 4
successive baths, at 1 hour intervals, in
0.15M phosphate buffer, at 4 °C.

Pieces were further postfixed in 1 %
osmic acid (OsO$_4$) in 0.15M phosphate
buffer, pH 7.2, for 90 min. at 4 °C, and then
washed 3 times, for 15 min., in the same
phosphate buffer.

Liver tissue fragments were then
submitted to a dehydration process, by
keeping them in acetone baths with
successively increasing concentrations (30 %,
50 %, 70 %, 80 %, 90 % and three times
100 %), 30 min in each bath.

Samples were gradually infiltrated
with synthetic resin Epon 812, in mixtures
of 1:3, 1:1 and 3:1 epoxicid resin:acetone,
and finally included in special capsules.

The capsules were kept in a
thermostat at 50 °C for 72 hours, to let the
resin polymerize.

The resin blocks containing the
samples were then shaped. For electron
microscopy, ultrathin sections of 30-60 nm
were obtained with a Leica UC6
ultramicrotome, with a Diatome Ultra 35°
diamond knife, and then collected on
electrolytic grids with 100-200 Mesh, and
double-contrasted with a 50 % alcoholic
solution of saturated uranyl acetate, and
lead citrate in distilled water, pH 12
(Craciun and Horobin, 1989; Toader, 1996;
Florea and Craciun, 2012).

Finally, the sections were examined
under a TEM Jeol Jem 1010 electron
microscope, at Center of Electron
Microscopy of Babes-Bolyai University,
Cluj-Napoca. Images were captured with a
Mega Wiew III camera.

These methodologies are consistent
with the classical methods widely used in
transmission electron microscopy (TEM)
(Ploaie and Petre, 1979; Weakley, 1981;
Hayat, 2000; Pavelka and Roth, 2005).

Ultrastructure of the control liver
Examination of normal liver tissue
revealed a parenchymal structure with
hepatocytes arranged in regular cords. Each
hepatocyte had one (Figs. 1a, 1c), or
occasionally two (Fig. 1b) spheric nuclei,
with a diameter of 5-7 µm and an uniform
outline.

The nuclei were predominantly
euchromatic, with the heterochromatin
dispersed in karyolymph as a network of
small electrondense blocks, in a fine layer
along the inner leaflet of the nuclear
anvelope (Fig. 1).

Nuclear pores, having a lower
electrondensity than the neighboring
heterochromatin, appeared clearly delimited
(Pribac et al., 2010).
Geometric morphometric analysis

The computerized geometric analysis of binary images was made using the Image J software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) (http://imagej.nih.gov/ij) together with MRI Cell Image Analyzer (MRI-CIA) software, developed by the Montpellier RIO Imaging Facility (CNRS) (http://www.mri.cnrs.fr/index.php?m=38) (Bäcker and Travo, 2006).

Let us consider a set of thirty digital images corresponding for the hepatocytes in normal rat livers, randomly selected from a database (Pribac et al., 2010).

After correction of the digital images, by contrast adjustment and spatial filtering, shape outlines (the plasma membrane, the nuclear anvelope and the cell nucleolus) were extracted from binary images with a classical contour-extraction method.

With area selections, the following geometrical parameters and numerical descriptors were determined: Area, Center of Mass, Perimeter, Bounding Rectangle, Fitted Ellipse, Feret’s Diameter, Skewness and Kurtosis.

Details of the representative size and shape parameters, as well as the intensity statistics, used to obtain information on the cytoarchitectonic characteristics complexity are given in the Appendix.

Statistical analysis

After image processing and analysis with ImageJ and MRI Cell Image Analyzer, all the raw data were statistically analyzed.

Descriptive statistics were calculated for hepatocytes in each group and the obtained average results were expressed as mean value and standard deviation.

It was found that the average values of the size and shape parameters followed a normal distribution.

The average values of the size and shape parameters presented are statistically highly significant (p < 0.01).

Results and discussions

We evaluated the representative size and shape parameters by the three criteria: fidelity, capture of biologically relevant details and human interpretability. The obtained average results were expressed as (average ± standard deviation).

A summary of the obtained results are presented in the tables given below.

In Table 1 and Table 2, the following specifications are used:

(Cell) = shape outline of plasma membrane;
(Nucleus) = shape outline for nuclear anvelope;
(Nucleolus) = shape outline for nucleolus.
### Table 1. Results of the evaluation of representative size and shape parameters of the hepatocytes (average ± standard deviation).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Shape type and values</th>
<th>(Cell)</th>
<th>(Nucleus)</th>
<th>(Nucleolus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area [µm²]</td>
<td>288.386 ± 51.147</td>
<td>39.9545 ± 8.826</td>
<td>2.3010 ± 0.5382</td>
<td></td>
</tr>
<tr>
<td>Perimeter [µm]</td>
<td>66.9189 ± 8.3740</td>
<td>23.1746 ± 3.4586</td>
<td>5.6371 ± 0.6478</td>
<td></td>
</tr>
<tr>
<td>Major [µm]</td>
<td>22.9051 ± 2.668</td>
<td>7.7492 ± 0.9833</td>
<td>1.8433 ± 0.2247</td>
<td></td>
</tr>
<tr>
<td>Minor [µm]</td>
<td>16.0381 ± 2.2456</td>
<td>6.4590 ± 1.2807</td>
<td>1.5736 ± 0.1812</td>
<td></td>
</tr>
<tr>
<td>Feret [µm]</td>
<td>24.7299 ± 3.639</td>
<td>7.8895 ± 0.9896</td>
<td>1.8984 ± 0.1970</td>
<td></td>
</tr>
<tr>
<td>FeretAngle [°]</td>
<td>27.7424 ± 36.703</td>
<td>54.9500 ± 23.805</td>
<td>115.0125 ± 45.251</td>
<td></td>
</tr>
<tr>
<td>MinFeret [µm]</td>
<td>16.7456 ± 23.387</td>
<td>6.5221 ± 1.2813</td>
<td>1.5946 ± 0.1642</td>
<td></td>
</tr>
<tr>
<td>Width [µm]</td>
<td>23.8500 ± 4.1846</td>
<td>6.8818 ± 1.3227</td>
<td>1.6792 ± 0.1796</td>
<td></td>
</tr>
<tr>
<td>Height [µm]</td>
<td>18.018 ± 3.5784</td>
<td>7.4462 ± 0.9496</td>
<td>1.7773 ± 0.2300</td>
<td></td>
</tr>
</tbody>
</table>

In intensity statistics, each color was defined as a value between 0 and 255, where 0 is the darkest (black) and 255 is the lightest (white). Higher values usually mean lighter pixels and lower values mean darker pixels.

The geometric morphometric analysis was in agreement with the ultrastructural observations. These analyses were performed using 2D representations of 3D biological shapes.

In 3D shape analysis, the cell surfaces can be represented implicitly (e.g., level sets) or explicitly (e.g., triangle meshes).

### Table 2. Intensity statistics evaluation for hepatocytes corresponding to the digital images processed in Table 1 (average ± standard deviation).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Shape type and values</th>
<th>(Cell)</th>
<th>(Nucleus)</th>
<th>(Nucleolus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Gray Value</td>
<td>157.865 ± 20.289</td>
<td>169.201 ± 16.891</td>
<td>85.998 ± 9.0617</td>
<td></td>
</tr>
<tr>
<td>Standard Dev.</td>
<td>29.5712 ± 8.9270</td>
<td>43.710 ± 12.6949</td>
<td>21.435 ± 1.9532</td>
<td></td>
</tr>
<tr>
<td>Modal Gray Value</td>
<td>153.4 ± 19.857</td>
<td>203.400 ± 15.4370</td>
<td>74.75 ± 4.272</td>
<td></td>
</tr>
<tr>
<td>Min Gray Level</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Max Gray Level</td>
<td>255</td>
<td>247</td>
<td>186</td>
<td></td>
</tr>
<tr>
<td>XM</td>
<td>25.2617 ± 8.0824</td>
<td>25.2670 ± 8.1555</td>
<td>17.4465 ± 6.2305</td>
<td></td>
</tr>
<tr>
<td>Integrated Density</td>
<td>44708.1 ± 2749.7</td>
<td>6683.368 ± 1801.1</td>
<td>194.551 ± 28.0811</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>156.20 ± 20.8135</td>
<td>183.200 ± 14.0961</td>
<td>83.000 ± 9.8319</td>
<td></td>
</tr>
<tr>
<td>Skewness</td>
<td>0.3707 ± 0.1544</td>
<td>-0.7079 ± 0.1605</td>
<td>0.8737 ± 0.1050</td>
<td></td>
</tr>
<tr>
<td>Kurtosis</td>
<td>1.0280 ± 0.2544</td>
<td>-0.5557 ± 0.4570</td>
<td>1.2667 ± 0.3200</td>
<td></td>
</tr>
</tbody>
</table>

One fundamental difference between the 2D and 3D shape analysis is that the methods for bringing volumetric data of shapes are more difficult in three dimensions.

### Conclusions

Computerized geometric morphometric method for shape analysis is a useful tool for investigation of healthy rat liver and allows numeric evidence of cell shape complexity and of the cytoarchitectonic characteristics.

Our results suggest that the hepatocytes shape complexity can be analyzed using a set of size and shape parameters in a highly effective, reproducible, accurate and statistically powerful way.

New facts provided by the geometrical evaluations, as quantitative feature of cell morphology, offer more relevant parameters in analyzing rat hepatocytes.
These size and shape parameters allow a sensitive characterization of very subtle variations in rat hepatocytes form that could remain undetected when using traditional particle sizing techniques.

This analysis method of rat hepatocytes shapes is computationally simple, with good and reproducible results across data sets, which make it a valuable tool for cell biologists and microscopists.

Acknowledgements

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Appendix

In our study, MRI Cell Image Analyzer (MRI-CIA) software, developed by the Montpellier RIO Imaging facility (CNRS)(http://www.mri.cnrs.fr/index.php?m=38) was used to determine the size and shape parameters of the given shape data.

With area selections, the following geometrical parameters and numerical descriptors were determined: Area, Center of Mass, Perimeter, Bounding Rectangle, Fitted Ellipse, Feret’s Diameter, Skewness and Kurtosis.

The size and shape parameters are defined as following:
1. Area: The surface of the region of interest (ROI), measured in [$\mu m^2$].
2. Perimeter: The length of the outside boundary of the ROI, measured in [$\mu m$].
3. Width: The width of the bounding box of the ROI (the smallest rectangle enclosing the selection).
4. Height: The height of the bounding box of the ROI.
5. Major: The length of the major axis of the best fitting ellipse. The ellipse has the same area, orientation and centroid as the original selection.
7. Angle: The angle of the major axis of the best fitting ellipse against the x-axis of the image.
8. Feret: The longest distance between two points on the boundary of the ROI, also known as maximum caliper. The length of the object’s projection in the X (FeretX) and Y (FeretY) direction is also displayed.
9. FeretAngle: The angle of the Feret diameter with the x-axis of the image (0°-180°).
10. MinFeret: This is a measure of the particle's width. It is called the minimum caliper diameter as well. It is defined as the shortest distance between two parallel planes touching the particle on opposite sites, for any orientation of the particle.

The intensity statistics parameters are defined as follows:
1. Mean Gray Value: The average gray value within the ROI. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels.
2. Standard Dev. 1: The standard deviation of the mean gray value within the ROI.
3. Modal Gray Value: Most frequently occurring gray value within the ROI. This corresponds to the highest peak in the histogram of the ROI.
4. Min & Max Gray Level: Minimum and maximum gray values within the ROI.
5. XM: The x-coordinate of the center of mass, that is the brightness weighted average of the x-coordinates of the pixels in the ROI. The coordinates (XM and YM) are the first order spatial moments.
6. YM: The y-coordinate of the center of mass, that is the brightness weighted average of the y-coordinates of the pixels in the ROI. The coordinates (XM and YM) are the first order spatial moments.
7. Integrated Density: The integrated density is the sum of the gray-values of all pixels within the ROI.
8. Median: The median gray value of the pixels within the ROI.
9. Skewness: A measure of the asymmetry of the distribution of the gray values around the mean within the ROI. The third order moment about the mean.
10. Kurtosis: A measure of the "peakedness" of the distribution of the gray values around the mean within the ROI. The fourth order moment about the mean.
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