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# Quantification of Cell Subpopulations, Fractions of Dead Cells and Debris in Cell Suspensions by Laser Diffractometry

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## Summary

*Laser diffractometry was employed for size analysis in liver cell and blood cell suspensions to assess its suitability for characterizing cell populations. The method proved sensitive to detect subpopulations in liver cells (bimodal or trimodal distributions) and to quantify their volume fractions. Cell debris and aggregates of cells could also be quantified, dead cell populations recognized by their shift in the mean cell diameter. Laser diffractometry is therefore suitable for determining the quality of cell isolations (e.g. by liver perfusion) or for following alterations in cell populations during culture of cells in suspension. Analysis of human blood allowed differenciations to be made between thrombocytes and other blood cells. No peak separation was obtained for the populations of erythrocytes and granulocytes due to their similarity in size. Monocytes could not be detected due to their extremely low number in the blood indicating the limit of the method.*

## Zusammenfassung

*Quantifizierung von Zellsubpopulationen, Fraktionen von toten Zellen und Zelltrümmern in Zellsuspensionen mittels Laser-Diffraktometrie*

*Die Laser-Diffraktometrie wurde für die Größenanalyse in Leberzell- und Blutzellsuspensionen eingesetzt, um ihre Eignung zur Charakterisierung von Zellpopulationen zu untersuchen. Die Methode erwies sich als ausreichend empfindlich, um Subpopulationen in Leberzellen zu detektieren (bimodale oder trimodale Verteilungen) und um das Volumen der einzelnen Fraktionen zu quantifizieren. Auch Zelltrümmer und Agglomerate von Zellen konnten quantitativ bestimmt werden. Tote Zellen wurden durch ihre Verschiebung im mittleren Zelldurchmesser erkannt. Die Laser-Diffraktometrie ist daher geeignet, die Qualität einer Zellisolierung (z. B. durch Leberperfusion) zu bestimmen und um Veränderungen in der Zellpopulation während der Suspensionskultur von Zellen zu verfolgen. Die Analyse von Humanblut erlaubte es, zwischen Thrombozyten und anderen Blutzellen zu unterscheiden. Die Populationen der Erythrozyten und der Granulozyten waren aufgrund ihres geringen Größenunterschiedes nicht zu trennen. Monozyten konnten wegen ihrer extrem kleinen Zahl im Blut nicht detektiert werden. Dies zeigt die Grenzen der Methode.*

**Key words:** *Cell suspension · Laser diffractometry*

## 1. Introduction

Microscopy is one method to determine cell subpopulations and to quantify their fractions using cell size and morphological appearance. Microscopic examination is relatively time-consuming. For many applications it can be replaced by particle sizing methods when the cell populatins are very different in size. The traditional and very successful instrument is the Coulter counter (Coulter Electronics, Krefeld, FRG). The Coulter counter is based on resistance measurements, the height of electric impulses is correlated to the particle size. Similar instruments based on the Coulter principle are on the market, some use a different signal processing. The Cell Analyser System Casey (Schärfe System, Reutlingen, FRG) calculates the area under the impulse for size determination. Sizing measurements of cells have multiple applications. They can even provide information about cell death and replace staining methods (e.g. for epitheloid cell line E 19—1, [1]). The cells shrink or deteriorate leading to a detectable decrease in the mean diameter.

During the last years laser diffractometers are increasingly employed for size analysis as an alternative to the Coulter counter. They are attractive because they are easier to handle and the analysis is very rapid (measurement and mathematical analysis less than 30 s). The question however is to what extent can the new method be employed to characterize cell populations, the traditional domain of the Coulter counter. To assess the suitability of laser diffractometry we analysed human blood and various liver cell mixtures differing in quality (purity, dead cells, cell debris, cell aggregates).

## 2. Materials and methods

### 2.1. Materials

Liver cells were obtained by perfusion of rat liver using perfusion buffers and chemicals described in detail elsewhere [2, 3]. Blood was obtained from human volunteers and analyzed directly after venous puncture. All cell suspensions were diluted with physiological salt solution for laser diffractometer analysis.

### 2.2. Methods

#### 2.2.1. Isolation of liver cells (cell suspension I)

The cells were obtained by perfusion of the liver of young male Sprague-Dawley rats. After in situ perfusion with a calcium-free buffer for approximately 20 min, the liver was removed and in a closed-loop system perfused with a buffer containing collagenase and calcium (20 min). The proteolytic enzyme dispersed the liver, the intact cells were suspended in buffer and filtered to remove remaining crue pieces of liver tissue [2, 3], this cell suspension contained hepatocytes (parenchymal cells), nonparenchymal cells (Kupffer cells, endothelial cells), and cell debris (cell suspension I). The cell suspension I was divided in equal parts for isolation of hepatocytes (cf. 2.2.2.) and of nonparenchymal cells (cf. 2.2.3.)

#### 2.2.2. Isolation of hepatocytes (cell suspension II)

The cells from suspension I were washed three times by centrifugation (2 min, 15 g). The nonparenchymal cells remained in dispersion during this centrifugation at low g number, the hepatocytes were obtained as cell pellet and re-suspended in cell culture medium (cell suspension II). This suspension contained mainly hepatocytes and contamination by nonparenchymal cells and cell debris [2, 3].

#### 2.2.3. Isolation of nonparenchymal cells (cell suspension III)

To obtain nonparenchymal cells the enzyme pronase was added to cells from suspension I. Pronase lyses hepatocytes whereas the nonparenchymal cells are relatively resistent against this enzyme. After 1 h most of the hepatocytes were lysed and the nonparenchymal cells were obained by centrifugation (4 min, 400 g). The cells localize in the pellet, the debris remain in dispersion. The pellet was resuspended in cell culture medium (cell suspension III [4]. The suspension contained mainly Kupffer cells, endothelial cells, and contamination by hepatocytes and cell debris. Most of the hepatocytes were dead (> 99 %, determined by Trypan blue staining).

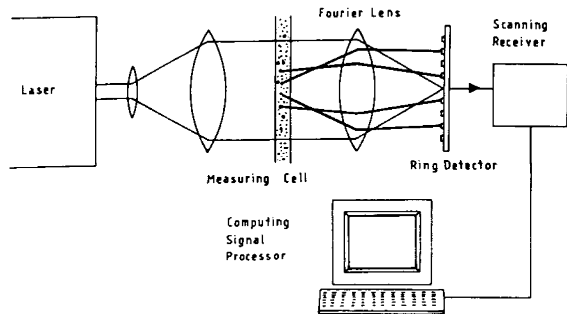


*Fig. 1:* Set up of laser diffractometer. The laser light is diffracted from the suspended particles forming a Fraunhofer diffraction pattern on a multi-element detector. The form of the pattern is size-spezific, its intensity correlates with the particle number.

#### 2.2.4. Determination of size distribution by laser diffractometry

The laser diffractometer is based on the diffraction of laser light from spherical particles (Fraunhofer diffraction) [6—8]. The particles are suspended in a transparent liquid (e.g. water, organic liquids), an extended laser beam passes through the sample cell and the diffracted laser light is detected by a multi-element ring detector (Fig. 1). The diffraction pattern is size-specific, its intensity is a function of the particle number. The superposition of different size-specific patterns each varying in its intensity as a function of the particle number can be mathematically resolved to calculate a volume distribution [9, 10]. The theory is based on spheres. The method is therefore less suitable for rod-like or needle-shaped particles but can be employed for spherical cells.

Measurements were performed in physiological salt solution using the suspension cell of the Sympatec laser diffractometer (Sympatec, Clausthal-Zellerfeld, FRG). Cell suspension was added until the instrument indicated optimum measurement conditions (diffraction intensity). For the measurement of the liver cell suspensions a 50 mm lense was used corresponding to a measuring range of 0.5 to 100 µm. Human blood was measured using a 20 mm lense corresponding to a measuring range of 0.1 to 74.5 µm.

## 3. Results and discussion

### 3.1. Characterization of cell suspension I (cf. 2.2.1.)

Two cell preparations (A, B) differing in quality were investigated. The size distribution curve of preparation A exhibits 3 peaks at approx. 3, 10, and 28.0 µm (Fig. 2A). The small peak represents the cell debris, the peak at 10 µm the fraction of nonparenchymal cells which are reported to possess a size between approx. 8 and 11 µm. Hepatocytes are in the range of 25 to 35 µm leading

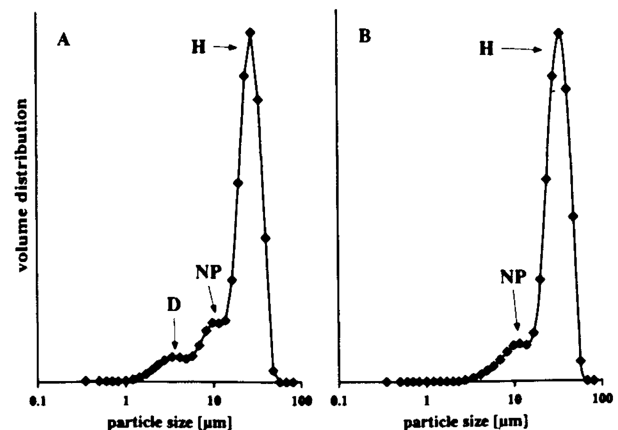


*Fig. 2:* Volume distributions of cell suspensions I obtained from liver perfusion (preparation A: *left;* preparation B: *right;* H = hepatocytes, NP = nonparenchymal cells, D = debris).

*Table 1:* Volume fractions of cell populations and contamination by debris in cell suspension I isolated from rat liver.

| Preparation | Debris (0.45—4.5 μm) | Nonparenchymal cells (4.5—15.0 μm) | Hepatocytes (15.0—43.5 μm) |
|---|---|---|---|
| A | 6.8 % | 17.0 % | 76.2 % |
| B | 0.9 % | 11.0 % | 88.1 % |

to the peak at 28.0 μm. The curves are volume distributions which explain the relatively large area under the peak of the hepatocytes. The hepatocytes are 60 % in number of the liver cells [11] but approx. 80 % in volume. This is well in agreement with the 76 volume % of the hepatocyte peak (Table 1). The peak of the debris is relatively large in preparation A. During preparation B less cells were destroyed during the dispersion of the liver. The peak of the debris is distinctly smaller (Fig. 2B), the area dropped from 6.8 to 0.9 % (Table 1). Therefore laser diffractometry provide information about the quality of the preparation. Quality being defined as percentage of total cells in contrast to debris. In addition, the fraction of a subpopulation in the total cell suspension can be determined (cf. 3.3.).

### *3.2. Characterization of cell suspension II (hepatocytes)*

A relatively large fraction of debris and nonparenchymal cells remained after centrifugation in the cell suspension II. There are still distinct peaks for debris and nonparenchymal cells (Fig. 3A). Centrifugation improved the purity of the hepatocyte population. However, the isolation A was less successful than isolation B as indicated by the volume percentage of hepatocytes (88.1 and 92.6 %, respectively, Table 2). This is also reflected by the missing debris peak and the relatively small nonparenchymal peak in preparation B (Fig. 3B).

### *3.3. Characterization of cell suspension III (nonparenchymal cells)*

The volume distribution of cell suspension III (preparation A) exhibits 3 large peaks, the largest peak at 23.5 μm representing hepatocytes (Fig. 4A). The peak of nonparenchymal cells located at approx. 9 μm is relatively small indicating no successful purification of the cell population. There is also a third fraction above 50 μm. This fraction consists of cell aggregates formed during the purification treatment. These aggregates could be identified as dead hepatocytes by Trypan blue staining. The peak of the hepatocytes shifted from 28.0 to 23.5 μm. This indicates that the cells are dead which could also be confirmed by Trypan blue staining. The cells shrink or deteriorate leading to a decrease in cell diameter. The separation of cells from debris was relatively high as indicated by the small tail of the volume distribution curve in the range up to 4 μm (5.7 % debris, Table 3). The debris resulted from hepatocytes lysed by pronase treatment.

In contrast, the isolation of nonparenchymal cells in preparation B was more successful as indicated by the large peak of the volume distribution curve at 7 μm (Fig. 4B). The volume fraction increased from 21.7 to 45.5 % at simultaneous reduction of the hepatocyte fraction (from 56.9 to 23.0 %, Table 3). However, the separation of cells from the debris was less successful compared to preparation A. Twice the fraction of debris remained in the cell suspension after centrifugation (Table 3). Further purification of the nonparenchymal cells can be achieved by cell culture techniques (e.g. adhesion to culture dishes and washing off of nonadherent cells and debris). Preparation B is the best preparation with regard to the yield of nonparenchymal cells.

### *3.4. Analysis of human blood*

Analysis of human blood yielded a bimodal volume distribution (Fig. 5). A small peak at 3 μm represents the thrombocytes (diameter 2—4 μm, 150 000—400 000 per μl [5]). The large peak were the erythrocytes (6—8 μm [5]) including the other blood

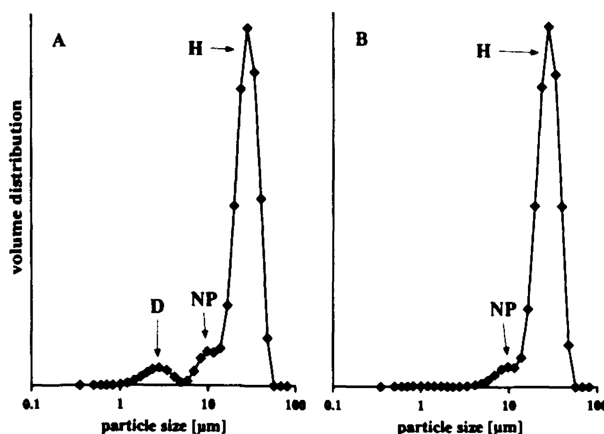


*Fig. 3:* Volume distributions of cell suspensions II obtained after purificating cell suspensions I (preparation A: *left*; preparation B: *right*; H = hepatocytes, NP = nonparenchymal cells, D = debris).

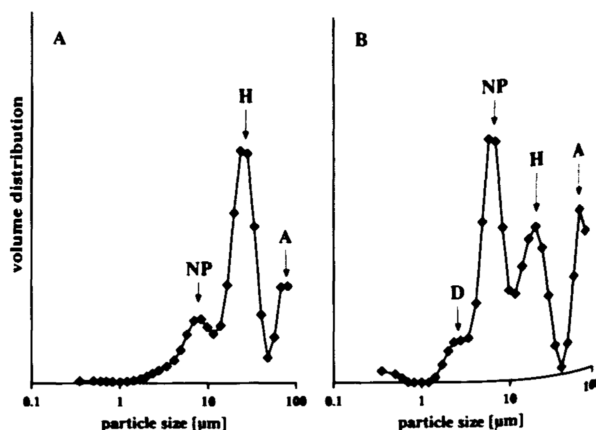


*Fig. 4:* Volume distributions of cell suspensions III obtained after pronase treatment and centrifugation of cell suspension I (preparation A: *left*; preparation B: *right*; NP = nonparenchyma cells, H = hepatocytes, A = agglomerates, D = debris).

*Table 2:* Volume fraction of hepatocytes and contamination by debris before purification (cell suspension I) and after centrifugation treatment to isolate hepatocytes (cell suspension II).

| Preparation | Hepatocytes | | Debris | |
|---|---|---|---|---|
| | before centrifugation | after centrifugation | before centrifugation | after centrifugation |
| A | 76.2 % | 88.1 % | 6.8 % | 5.2 % |
| B | 88.1 % | 92.6 % | 0.9 % | 0.6 % |

*Table 3:* Volume fractions of nonparenchymal cells and hepatocytes and contamination by debris and agglomerates after pronase treatment of cell suspension I and centrifugation to isolate nonparenchymal cells (cell suspension III).

| Preparation | Debris (0.45 to 4.5 μm) | Nonparenchymal cells (4.5 to 15.0 μm) | Hepatocytes (15.0 to 43.5 μm) | Agglomerates (> 43.5 μm) |
|---|---|---|---|---|
| A | 5.7 % | 21.7 % | 56.9 % | 15.7 % |
| B | 13.4 % | 45.5 % | 23.0 % | 18.1 % |

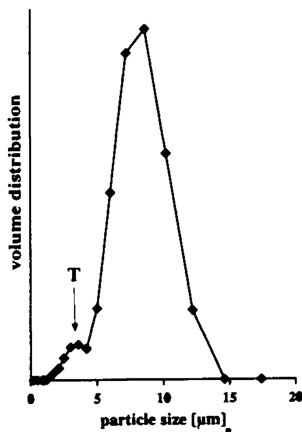


*Fig. 5:* Volume distribution of human blood analyzed by laser diffractometry (small peak: thrombocytes (T), large peak: erythrocytes, lymphocytes, and granulocytes).

cells being in the range 8—12 µm (granulocytes, lymphocytes [5]). All detected cells were below 12.2 µm. This means that the low fraction of larger sized monocytes (14—17 µm) was not detected. The number of 140—700 monocytes per µl blood is too low compared with the large number of erythrocytes and therefore below the detection limit of the laser diffractometer. A higher resolution of the distribution between 6 and 12 µm cannot be obtained by laser diffractometer. For this application a Coulter counter appears more suitable (e.g. use of a 30 or even 15 µm capillary).

### 3.5. Further applications

A major advantage of the method is the use of any dispersion medium which is transparent for light. Residues of electrolytes from the cell isolation process or cell culture medium interfere with assays based on conductivity measurements (e.g. Coulter counter). Agglomerates in the range of 50 µm are no problem for the laser diffractometer but will block the 50 µm capillary of a Coulter counter. This requires the use of a larger capillary such as 100 µm, setting the lower detection limit to 2 µm. A part of the debris cannot be detected any more whereas the laser diffractometer has a measuring range from 0.5 to 87.5 µm. The upper limit for a Coulter counter 100 µm capillary is about 80 µm.

Advantages of the Coulter counter are the determination of an absolute cell number per volume unit and its sensitivity to detect a few large cells in a large population of smaller cells. A Coulter counter appears therefore more suitable for detecting the low number of monocytes (14—17 µm, 140—700 monocytes per µl blood) in human blood with a dominating number of red blood cells (6—8 µm, 4—6 million per µl, [5]). A number distribution can, however, also be obtained by a laser diffractometer using a defined volume of cell suspension and converting the volume distribution mathematically to a number distribution. However, the mathematical conversion is less precise than the direct measurement of the number distribution (Coulter counter).

## 4. Conclusions

Laser diffractometry is a very rapid method to determine the composition of mixed cell populations and possible contamination by debris or cell agglomerates. The volume distributions obtained were in good agreement with literature data. In contrast to conductivity-based assays, salts from the isolation process or cell culture medium do not interfere with the assay. Laser diffractometry is as a multiple particle-counting technique, however, less sensitive in detecting a few large cells in a cell suspension containing a large number of small cells (e.g. monocytes in human blood with many erythrocytes) or vice versa.

## 5. References

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