Computer-assisted centrifugal elutriation.
I. Detection system and data acquisition equipment

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To optimize cell separations by centrifugal elutriation, we constructed an on-line computer-controlled multiparametric light-scatter system. A bypass sample flow, at the outlet of an elutriation rotor, is hydrofocussed and three scatter parameters of each cell are determined up to a maximum of 15 000 cells/second. The 18-bit representation of the parameter values are cumulatively stored by means of a direct memory access interface. The histogram memory is continuously displayed to provide information on the number and type of cells that are elutriated. A special purpose operating system, implemented on a stand-alone computer configuration, allows a high data-acquisition rate and ample data processing capacity. In addition, a local network driver was constructed to facilitate off-line detailed analysis of the data. The equipment is well suited to monitor the centrigural elutriation process. The flexibility of the system allows an extension of the monitor to computer-controlled elutriation.

Cell separation; Centrifugal elutriation; Multiparametric light-scatter analysis; Stand-alone computer; Operating system; Local network

1. Introduction

Centrifugal elutriation (CE) is a powerful physical cell separation technique by means of which differences in sedimentation velocity of human peripheral blood cells are exploited to isolate various types of cells [1–3]. It is well suited for separating large numbers of mononuclear cells into lymphocytes and monocytes [4–7], or isolating subpopulations of monocytes that differ in function and maturation/differentiation stage [8]. Currently, CE is widely used to purify inhomogeneous cell populations, for both clinical and fundamental research applications [8–10]. However, until now separations have been carried out in a rather arbitrary manner, since analysis of elutriated cells is performed after separation. Therefore two major disadvantages are related to the practical application of CE.

First, during elutriation no information is present on the progress of the separation process. This implies that optimization of the CE technique can only be attained by means of trial-and-error methods [11–14]. Secondly, the morphological differentiation and characterization of the various eluted fractions is an extremely time-consuming process, thus limiting the practical applicability of CE. Cell identification equipment, with a data acquisition unit, interfaced to the CE rotor might overcome these disadvantages. However, since CE is generally applied to prepare homogeneous cell populations for further biological ex-

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periments, no biochemical modification (e.g. staining) of the cells under separation is allowed. This implies that we are limited to nondestructive remote sensing of cells that emerge from the rotor.

The present communication reports on the construction of an on-line real-time computer-controlled light-scatter apparatus to monitor and optimize cell separations performed by CE. A separate paper describes the off-line software developed to analyze the data available from elutriation experiments. This equipment allows on-line monitoring of the separation process and instant analysis of the isolated fractions.

2. Background

In this section, a concise description of the CE technique, as well as a brief review of the elastic light-scatter properties of a single cell in flow, is presented. Concurrently, the disadvantages related to CE, and the considerations with respect to the design of the detection system, are discussed.

In CE—or counterflow centrifugation—a fluid is pumped into the center of a centrifuge rotor through a rotating seal. The fluid moves through internal tubing into a divergent chamber situated in the rotor. At the end of this chamber the flow reconverges into a small tube and leaves the system through the rotating seal. As described previously, concentration of the cells is accomplished by means of a second rotor in series with the first one [15]. A mononuclear cell suspension, prepared by a blood component separator [16], is injected into the first rotor and moves through the system into the separation chamber. The separation chamber coerces the cells to rest, relative to the rotor, at a point where the outward-directed centrifugal forces are balanced by the inward-directed fluid dynamics. The cell population redistributes itself along the separation chamber in accordance with the physical properties (volume and density) of each cell (Fig. 1) [1–3,17]. Fractionation is obtained by stepwise decreases of the speed of the first rotor, while the speed of the second rotor remains at a constant value to collect and concentrate the eluted cells. Further details of the CE equipment, the sample introduction and
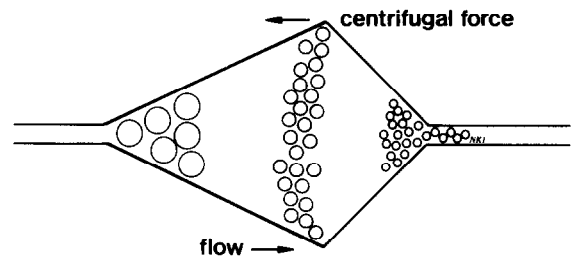

Fig. 1. Redistribution of mononuclear cells in a centrifugal elutriation rotor chamber.

the flow distribution unit were described previously [15].

Inherent in the CE technique are several factors that might influence the resolution capacity of the cell separation. Optimization of the CE system is obtained by investigating these resolution-improving parameters. For instance, the influence of temperature of the fluid [11,12,14], the shape of the elutriation chamber [14,17] and cell load and density of the elutriation fluid [18], were studied and modified to optimize the separation process. Morphological characterization of the fractions is always performed after separation by staining the cytocentrifuge preparations and by electronic sizing. This implies that no information is available during the separation process. In addition, the differentiation is hampered by subjective interpretation (microscopic analysis), or poor differentiation capacity (electronic sizing cannot always discriminate between various subpopulations of human blood cells). An on-line detection system will improve the interpretation of this type of experiment. Such a system reveals information on the characteristics of the separation process, and reliable fractionation criteria (e.g. fractionation time and speed of the rotor) are within reach.

In a previous investigation, Mulder et al. constructed a one-parameter monitor to study the purification of monocytes by means of CE [19]. Although this monitor could not discriminate between the various (sub)populations (e.g. monocytes/granulocytes), and did not allow further analysis of the data, the results clearly demonstrated the applicability of such a monitor system. Therefore we designed a light-scatter device, interfaced to the CE system, which facilitates detection

of all significant subpopulations in human peripheral blood. Most light-scatter instruments (e.g. flow cytometers) used for the differentiation of biological cells, measure forward scatter (FS) vs. fluorescence or FS vs. side scatter (SS) [20–24]. Recently, however, we showed numerically that optimization of the detection can be obtained by the simultaneous measurement of FS, SS and back scatter (BS) [25]. Therefore, a three-parameter light-scatter monitor was designed and interfaced to the CE system.

3. Design considerations

In the previous sections we emphasized the necessity of an on-line three-parametric light-scatter apparatus to monitor CE experiments. The equipment should meet certain specific conditions, which are discussed in this section.

Table 1 shows the statistics of a typical CE experiment in which mononuclear cells, isolated from peripheral blood of a human donor, are separated under standard conditions. Morphological characterization of the fractionated populations is established by May Grünwald Giemsa staining of cytocentrifuge preparations and subsequent microscopical analysis [15,16]. The data exhibit a characteristic maximum number of cells in the lymphocyte fractions (at a rotor speed of 3100 rpm). Since we are interested in the maximum number of cells *per second*, the elutriation profile of the 3100-rpm fraction is measured (by counting each minute the number of cells that are eluted from the first rotor). The results show that a new equilibrium is established within 2 min (Fig. 2). Subsequently, the cell rate slowly decreases and the nonequilibrium cells are eluted from the separation chamber. From Table 1 and Fig. 2 it can be concluded that the maximum cell rate expected is approximately 1×10^6 cells/s. Consequently, three parametric detection of a significant number of the eluted cells ($\sim 0.3\%$) necessitates a high-speed data-acquisition system.

The biological spread causes a homogeneous broadening of the light-scatter signal. Therefore, a limited (e.g. 6-bit) resolution of each parameter is sufficient to obtain a realistic representation of each detector signal.
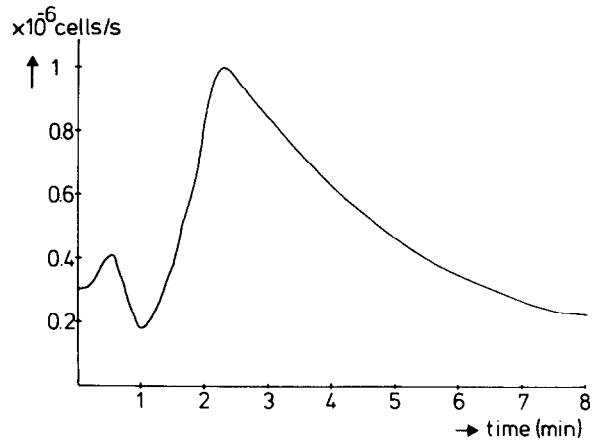

Fig. 2. Elutriation profile of a lymphocyte fraction.

On account of the large amount of data (0.3% of 10^6 cells, represented by 18 bits, equals-60 Kbits per second), a real-time histogramming direct memory access (DMA) interface has been designed. This interface imposes a memory organization where each address is defined by an 18-bit combination of the detected signal. Each new event (i.e. a detected cell) triggers the interface, and the contents of the corresponding memory location are subsequently increased by 1 (up to a maximum of $2^{16} - 1$: due to the homogeneous broadening and the total number of detected cells, the number of cells in a single channel is expected not to exceed this maximum).

Since the system should provide direct information on the elutriation process, the data, stored by means of the DMA interface, are graphically displayed on a CRT screen. To investigate the data more closely, a communication between the on-line memory and a host computer[1], is necessary. Serial transmission of the complete computer memory is too slow, since 512 Kbytes of data ($2^{18} \times 16$) results in a transfer time of almost 10 min. A parallel link, on the other hand, limits the distance between the host computer and the monitor system. To avoid these difficulties, a local area network was used. Subsequently, a network driver

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[1] LoVME 68010 (Microproject BV, The Netherlands), running the Unix operating system (Unix is a trademark of Bell Labs).

TABLE 1

Statistics of a centrifugal elutriation experiment of 700×10^6 mononuclear cells with an elutriation time of 8 min/fraction and a flow rate of 18 ml/min

Fraction	Rotor speed (rpm)	Total number of cells ($\times 10^{-6}$)	Percentage of cells in fraction				Number of cells in fraction ($\times 10^{-6}$)				Average number of cells per minute ($\times 10^{-6}$)			
			E	L	M	G	E	L	M	G	E	L	M	G
1	3500	115	90	10			103.5	11.5			12.9	1.5		
2	3300	60	50	50			30	30			3.8	3.8		
3	3100	225	20	80			45	180			5.6	22.5		
4	3000	25	15	80	5		3.8	20	1.3		0.5	2.5	0.2	
5	2900	8	3	57	35	5	0.2	4.5	2.6	0.5		0.5	0.3	0.1
6	2900	7	3	20	72	5	0.2	1.4	5.3	0.4		0.2	0.7	0.1
7	2800	75	3	5	90	2	2.3	3.8	67.5	1.5	0.3	0.5	8.4	0.2
8	2700	35	3	4	86	7	1.1	1.4	30.1	2.5	0.1	0.2	3.8	0.3
9	2650	50	3	11	47	39	1.5	5.5	23.5	19.5	0.2	0.7	2.9	2.4
10	stop	100	3	4	11	82	3	4	11	82	0.4	0.5	1.4	10.3

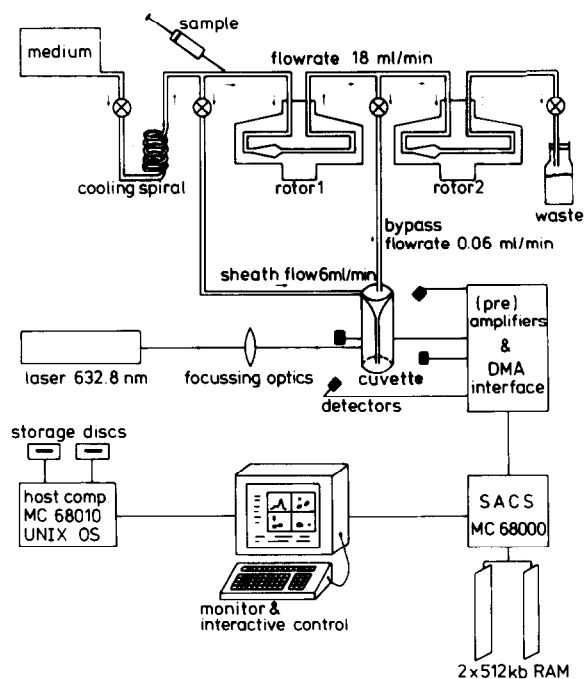E, erythrocytes; L, lymphocytes; M, Monocytes; G, granulocytes.

Fig. 3. Outline of the complete computer-assisted centrifugal elutriation system. SACS, stand-alone computer system; OS, operating system.

was designed and implemented. Finally, simultaneous control of data acquisition, data flow and graphical representation required a special-purpose operating system (OS). In addition, an OS (with low-level control of the hardware) allows a hardware-independent software organization. This results in flexible soft- and hardware design. Consequently, modifications and/or extensions are easily implemented.

4. System description

An outline of the complete CE monitor is shown in Fig. 3. The details of the signal processing unit and the data acquisition will be discussed in this section.

The CE system consists of two JE-6 elutriation rotors, connected in series and equipped with standard separation chambers[2]. To monitor the

cells elutriated from rotor 1, a shunt is inserted between the two rotors. The flow rate of the bypass (0.05 ml/min) assures that a sufficient amount of elutriated cells can be studied without significantly diminishing the cell yield. The bypass sample flow is hydrofocussed into a commercially available cuvette[3] to facilitate the excitation of each single cell by means of a focussed laser beam [26]. To compensate for the additional flow circuit, and the related additional flow resistance, an extra flow controller is inserted in the waste flows. This allows tuning of the combined flow system and results in a stable sample flow.

Light from a He/Ne laser[4] is focussed by means of two crossed cylindrical lenses ($f = 60$ mm and $f = 20$ mm) to a laser spot of approximately 20 μm $\times$ 80 μm. The small deviation of the laser spot from the theoretical values (15 μm and 50 μm, respectively) is caused by inevitable misalignment and irregularities in the beam-shaping optics, but does not affect the optical detection [27]. The He/Ne excitation wavelength (632.8 nm) assures that no relevant absorption is present in the studied samples [28–30]; therore, the light attenuation is completely described by elastic light scatter [20]. The light scattered by each cell that enters the laser field is detected in the FS, SS and BS directions by a number of photovoltaic detectors[5]. A second SS detector is installed to compensate for possible variations in the hydrofocussed sample flow. The four BS detectors (2 $\times$ 5 mm) are centered around the incident laser beam. The light collecting angles are: FS, 1° to 7°; SS, 75° to 100°; BS, 160° to 180°, respectively. The geometry of the light-scatter detectors, in combination with the related acceptance angles, guarantee optimal detection of the light-scatter parameters of nucleated blood cells: FS is sensitive to cellular gross size, SS is mainly determined by the optical density of the cytoplasm and BS is exclusively related to the optical density of the nucleus [24,25].

The scatter signals of each cell are digitized to

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[2] Beckman Instruments, Palo Alto, CA, U.S.A.

[3] Ortho Diagnostic Systems, Belgium.

[4] 10 mW random polarized; Spectraphysics.

[5] FS: huv-1000bq; SS: huv-4000b: EG&G; BS: 4 BPY-11 detectors (Siemens) in series.
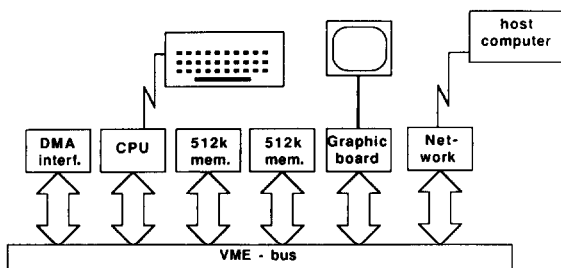
Fig. 4. Hardware configuration of the CACE equipment.

Fig. 5. Software structure of the stand-alone OS.

form a combined 18-bit address. By means of DMA, the contents of the address are increased by 1. This data acquisition technique is in contrast with regular techniques, where list mode facilities have been applied to detect and store multiparameter information [31]. The advantages of the simultaneous storage technique are evident: it requires only a minor on-line memory and all information (e.g. correlation between parameters), necessary for implementation of off-line parametric and non/parametric analysis [32], remains directly available. Communication between the interface, the central processor[6], the memory[7], the graphical board[8] and the network adaptor[9] is established by means of VME-bus logic (Fig. 4). In addition, cell rate, overflow occurrences and DMA activity are simultaneously measured and displayed.

To control the various processes that are active at the same time on the same processor, a time-sharing provision must be present. In addition, low-level access to various devices is necessary. Therefore, a special-purpose OS has been developed. Interrupt handling, process scheduling, device access, data manipulation as well as data representation, are controlled by this OS, the structure of which is shown in Fig. 5. The innermost (software) layer allows a process to wait for an event generated by another process, or an external event (e.g. interrupts activated by the operator). Consequently, administration of interrupts and synchronization of simultaneous processes are provided by this first layer. The

second layer of the OS is a uniform interface to several different hardware devices. It contains a number of procedures to perform (buffered) input and/or output (e.g. type-ahead facilities). The third layer implements the command interpreter and the (operator–computer) communication procedures. Application software, implemented as 'highest-level modules', facilitate straightforward modification and extension of the system. This stratified module structure is supported by several programming languages, e.g. ADA, PASCAL [33] and MODULA II. Since no ADA compilers were available, MODULA II was used as the most convenient language. It supports separate compilation, low-level access and has a strong modular concept. In addition MODULA II facilitates implementation of concurrent programming [34].

As described in the previous sections, elutriation is performed by decreasing the rotor speed and harvesting the eluted cells. The information of each fraction, stored in the memory, must be available for further statistical analysis. Therefore, the monitor is interfaced to a host computer by a commercially available hardware device. In the remaining part of this section we will give a concise description of the implementation of the local network on the stand-alone system.

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[6] FORCE sys68K/CPU-1.
[7] FORCE sys68K/DRAM-1.
[8] ELTEC GRAZ-1/68K.
[9] Microproject BV, The Netherlands, No. 2501-7969-6.

The structure of the local network is comparable to the structure of the OS. The physical, datalink and network layers, with their corresponding protocols, are part of the standard configuration of the hard- and software of the adaptor. Implementation of a network on the OS, however, requires a transport layer (i.e. network driver) [35]. This driver resets the adaptor, reads or writes a packet, handles interrupts and closes the adaptor. In addition, the driver supports access of simultaneous processes by means of virtual devices and (de)multiplexing communication. The software constituting the dump application resides in the top layer of the network structure. It allows the operator to dump the complete updated memory to the host computer. Comparable programs exist for reading packets of information from the host computer.

The complete software package (operating system, network and application) consists of 360 Kbytes of source code. After compilation and linking, 150 Kbytes of object code remains to be downloaded.

5. Status report

The reliability of computer-assisted centrifugal elutriation (CACE[10]), is primarily determined by the quality of the detection system. Especially, the detection of a single cell in flow is hampered by a number of experimental difficulties. We therefore studied the performance of the light-scatter set-up by injection of various well-defined particles (1–10 μm $\pm$ 10 nm) into the sample flow. For instance, the influence of the beam-stop in the FS direction has been studied. Experiments showed that an extremely small beam-stop with cylindrical shape, in addition to a small detection angle, in the FS directions results in an optimal correlation between FS and particle size. This is in agreement with recent theoretical and experimental results [36]. Furthermore, it was concluded from these experiments that reflections from daylight introduce noise to the SS signal. This is due to the extreme weak SS signal: approximately 10^{-10} of

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10 Patent applied for in the Netherlands.

the incident radiation (i.e. $\sim 10^{-9}$ mW). Therefore, a light-screening cone was mounted onto the SS detector. In addition, a second SS detector was introduced, in series with the first one, to account for insurmountable variations in the hydro-focussed sample flow. After establishment of the quality of the detection system, the linearity of the signal amplification with respect to the frequency of particles passing through the laser beam was studied. It was concluded that a maximum cell rate of approximately 15 000 cells/s is still within the specifications of the equipment (data not shown). Since DMA signal processing is applied, the *actual* cell rate limit is also 15 kHz. This implies that the data acquisition is suitable for efficient on-line sampling (interfacing to the CE rotors).

In a preliminary software configuration, the time required to produce 4 simultaneous scatter-plots exceeded 1 min. This was due to a memory-scanning module written in MODULA II. Therefore, in contrast to all the other modules, the scanning routine was rewritten in assembler code. Consequently, the current picture-producing time is as small as 15 s. Information on the CE process is therefore available within approximately 30 s (15 s additional delay is caused by the distance between the detection system and the elutriator rotor).

The merits of the network and its protocol were studied by sending and receiving a large number of 0.5-Mbyte packets. A minor adaption of the sending protocol resulted in a current (error-free) transfer time of 3.15 min for 512 kbyte of data. Since approximately 5 min are required to harvest the eluted cells from the second rotor and to initialize the next fraction, a transfer time of ~ 3 min is sufficient. However, further improvement of the speed of data transfer would contribute to the flexibility of the system (e.g. intermediate transfers). Therefore, the transfer protocols (i.e. the time-limiting modules) are still under study.

After establishment of the quality of the parts constituting the system, the equipment was assembled and interfaced to the CE rotors (Fig. 3). A number of separations were carried out to study the complete performance of the system. A flowchart representing the user-application mod-
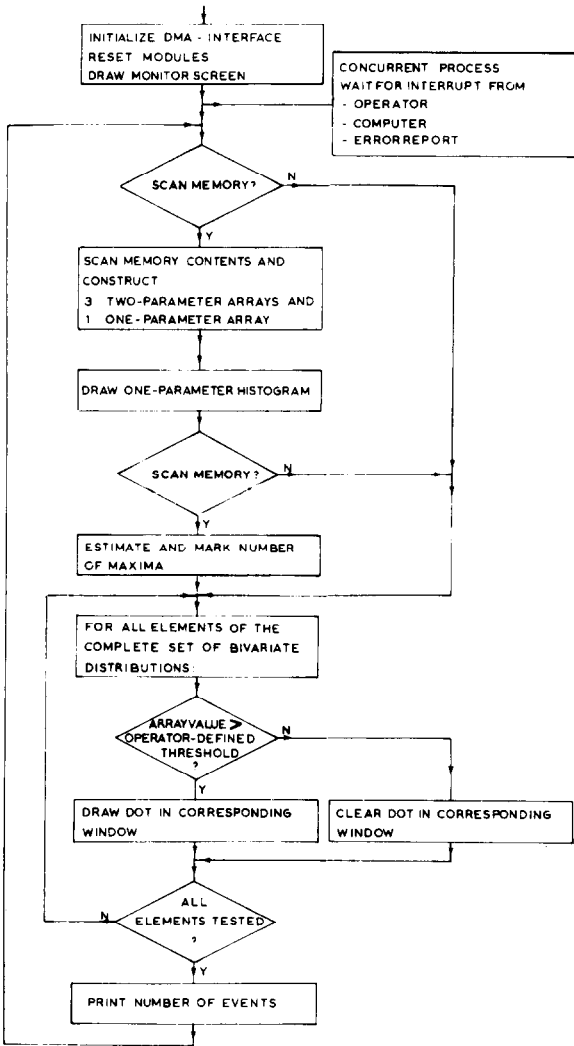
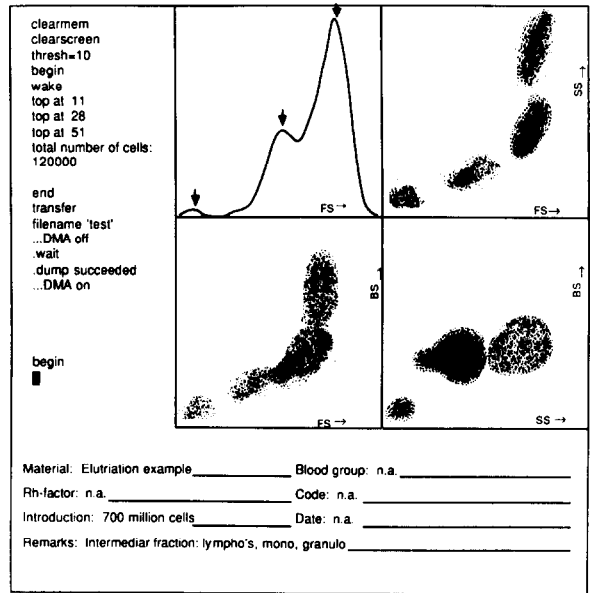Fig. 6. Flowchart of the application module, indicating the concurrent processes during a CACE experiment.

Fig. 7. A simulated screen-dump of a typical CACE experiment. The left window shows the communication of the operator with the computer. The bottom window displays the statistics of the experiment.

ule is shown in Fig. 6. Two concurrent processes are active in this module: one process reads the data and calculates the information for graphical representation, whereas the other process is waiting for a computer signal or an operator signal (interrupt). The result of a typical intermediate fraction, containing lymphocytes, monocytes and a small percentage of neutrophilic granulocytes, is shown in Fig. 7. It is observed that the various (sub)populations are well defined, and that the information available from the monitor facilitates tuning of the separation process.

6. Lessons learned

In the previous sections, the necessity and the design of a CACE system is described. The current level of performance and implementation of the equipment indicates that computerized multiparametric remote sensing of a single elutriated cell is well suited to study the CE process. The memory organization and the DMA interface allows high-speed 3-parametric data acquisition of the scatter parameters. In addition, the processor time can be used to manipulate the data, show the status of the separation process and communicate with the operator. The resolution of the scatter parameters (3×6 bits), assures an adequate representation of the (sub)populations of the cells that are elutriated. Higher resolution (e.g. 3×8 bits) will not significantly improve the scatter information. Moreover, the time required to produce an on-line scatter-plot would increase dramatically. The flexibility of this CACE system allows straightforward modification of the hardware devices and/or the user-defined software. This is

essential, since both the soft- and hardware are regularly adapted to fulfill the requirements of various types of experiments. In addition, the choice of an operating system allows a user-friendly communication between the operator and the numerous (active) processes of the computer. Therefore, the system can be applied in routine experiments such as preparative separations of a large number of human peripheral blood cells [7]. From these observations, it can be concluded that the system may facilitate both clinical and research applications.

The computer language MODULA II (a descendant of PASCAL) allows low-level access and is well suited for applications where hardware devices are interfaced. In addition, the module concept (especially the definition and implementation module facilities) and the unique procedure type facilitate the design of system software. Furthermore, compatibility and flexibility of the existing software is guaranteed by the modular structure of the software: modifications are easily implemented.

7. Conclusions and future plans

In this paper we report on the construction of a computer-assisted centrifugal elutriation device. Presently, we apply the system to investigate a number of CE parameters that could influence the resolution capacity of the separation process. For instance, the elutriation profile, the optimal elutriation time and the relation between the speed of the rotor and the separation of the various (sub)populations will be investigated. A better understanding of the fluid dynamics in the rotor chamber is within reach using this remote sensing technique. In the near future a number of modifications and/or extensions will be added to the system: to study the time-dependent inhomogeneity of an eluted fraction, intermediate dumping of the memory contents must be applied. Currently, however, the time needed for a complete data transfer is to long to allow this intermediate data transfer. Therefore, the (CRC) protocols of the network will be optimized. In addition, the top layer of the OS software will be modified to allow

more advanced graphical utilities. For instance, masked bivariate plotting capabilities and on-line software windows, to calculate changes in the eluted subpopulations, will be implemented. Downloading of the software into the stand-alone system is a time-consuming process (150 Kbytes of object code requires approximately 2 min loading time). Therefore, the complete software package will be stored into an EPROM. This allows direct execution of the software after (re)initialization of the hardware.

Recent theoretical considerations suggest that the anisotropy introduced by cellular membrane inhomogeneities and multiple scattering can be studied by measuring (de)polarization of the incident light [37,38]. The CACE system will be adapted in accordance with this observation.

The flexibility of the CACE system allows modification of the soft- and hardware to facilitate the design of a 'turn-key' computer-*controlled* centrifugal elutriation system. First, computer-controlled detection and regulation of the complete flow system and the rotor speed must be established. Next, an accurate on-line differentiation of the various subpopulations, and a hardware device controlling the rotor speed, must be designed. Finally, to facilitate routine application of such a system, autofocussing algorithms will be investigated [39].

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