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AQUATIC FLOW CYTOMETRY: ACHIEVEMENTS AND PROSPECTS. *M. RECKERMANN and F. COLIJN (eds.)*

Flow cytometry and integrated imaging*

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SUMMARY: It is a serious problem to relate the results of a flow cytometric analysis of a marine sample to different species. Images of particles selectively triggered by the flow cytometric analysis and picked out from the flowing stream give a valuable additional information on the analyzed organisms. The technical principles and problems of triggered imaging in flow are discussed, as well as the positioning of the particles in the plane of focus, freezing the motion of the quickly moving objects and what kinds of light sources are suitable for pulsed illumination. The images have to be stored either by film or electronically. The features of camera targets and the memory requirements for storing the image data and the conditions for the triggering device are shown. A brief explanation of the features of three realized flow cytometric imaging (FCI) systems is given: the Macro Flow Planktometer built within the EUROMAR MAROPT project, the Imaging Module of the European Plankton Analysis System, supported by the MAST II EurOPA project and the most recently developed FLUVO VI universal flow cytometer including HBO 100- and laser excitation for fluorescence and scatter, Coulter sizing as well as bright field and and phase contrast FCI.

Key words: flow cytometry, imaging, flash illumination, nanolite, LED-flash.

INTRODUCTION

Relating the results of flow cytometric measurement to the more or less known different species in a sample represents a serious problem. If free water samples are analyzed it is of great interest to know what kind of cells or organisms are hidden behind the abstract peaks shown on the screen of the flow cytometer. A more or less circumstancial method to visualize the objects represented in a histogram is to sort out the objects of a single- or multiparameter-range and to prepare the sorted organisms for a microscopic analysis. Such systems, however, are expensive and the additional preparation work is laborious and time consuming. A logical conclusion is to combine in the same instrument the abstract flow cytometric analysis and the uptake of images of objects of particular interest. Flow cytometric imaging (FCI), however, is a high speed process involving problems, which complicate its implementation. This paper gives a short introduction into principles and construction of such combined systems.

We started with FCI investigating technical aspects (Kachel, 1974; Kachel *et al*., 1990): we wanted to know how cells behave in the sensor region and how this behaviour influences the results of the FCM analysis. We have learnt from such investigations how flow forces ensure orientation and in some cases deformation of cells and that orientation of non spheric particles may strongly influence the precision of Coulter sizing and laser illuminated fluorescence analysis.

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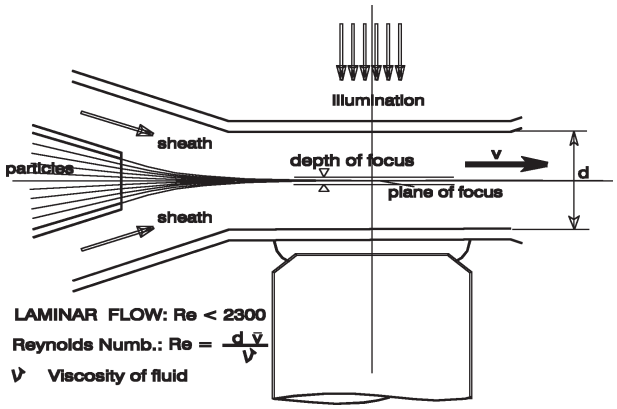

FIG. 1. – The conditions for laminar flow and hydrodynamic focusing; v: flow speed = moving speed of the objects, d: diameter of the flow path.

TECHNICAL PRINCIPLES AND PROBLEMS

Positioning the particles

The particles to be imaged have to be positioned in the plane of focus of the imaging optics. For typical microscopic systems the depth of focus depends on the resolution of the imaging optics and lies below 1 μm. By hydrodynamic focusing the particles are positioned within the limited depth of focus of the imaging optics (Fig. 1). Like in the most of the flow cytometric sensors, the particle stream is injected by a separate tube into a sheath flow which constricts the particles to a narrow stream. Its diameter is determined by the flow path constriction as well as the relation of the flow speeds of sheath and particle stream (Kachel *et al.,* 1980; Kachel *et al.,* 1990). In addition to the narrow radial extension, the stability of the position of the particle stream is of vital importance. Deviations of a few microns position the particles outside the depth of focus. Highly laminar, symmetric flow- and stabilized pressure conditions guarantee a steady position.

Freezing the motion of the objects

Particles in a FCM-sensor are moving with speeds of several m/s. Sharp images can only be obtained when the motion is frozen by short-time illumination of a pulsed light source or a short time shutter of the image uptake system. The time conditions for generating sharp images depend on the flow speed and the optical magnification, which defines the extension of the image on the camera target (Fig. 2). With v1 = 2m/s and assuming l_b = 0.25μm t_b = 125 ns. An additional condition from the pixel structure of CCD targets is $L_b < d_p$.

Suitable short time light sources are:

Pulsed Light Emitting Diodes (LED) emitting red light: $t_b > 100$ns;
Nanolite flashlamps, emitting Blue and UV light: $9\text{ns} > t_b < 100$ns;

Pulsed light sources emit high energy only for the duration of the short illumination time. They can be triggered easily and used with standard video cameras. LEDs are of small size, of low cost and need only simple electronics for operation. But presently the pulse duration and energy to be emitted is limited by physical and constructive reasons. Nanolite flash lamps are expensive, need more space but deliver extremely short pulses of high energy. Video cameras with shutters in the nanosecond range are expensive. Since the particles to be imaged appear randomly, the high energy illumination system has to be permanently switched on and could produce heat and bleaching problems in the transducer.

Image uptake and storing

In principle high image rates and high resolutions can be achieved by photo cameras and photographic film (Kachel *et al.,* 1979; Kachel *et al.,*

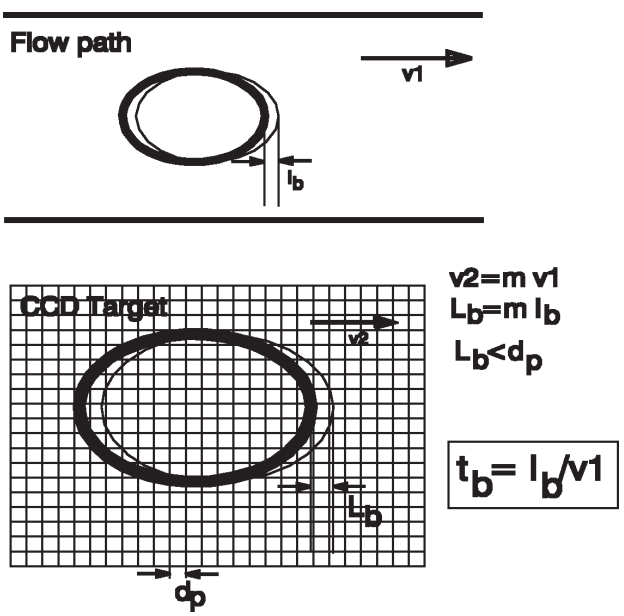

FIG. 2. – The relations of motional displacement and illumination time t_b; v1: speed of the object, v2: speed of the image of the object on the target, d_p: extension of a pixel on the target in the direction of movement, l_b: distance the object is moving per time unit, L_b: distance the image of the object is moving on the target per time unit, t_b: ilumination time allowing a motion of the image on the target < d_p

1980). But the circumstantial processing and archivation renders this medium unsuitable for routine use. Digital electronic images are immediately ready for evaluation without loss of quality and for storing in modern data banks. Thousands of images can easily be managed, reproduced and made available worldwide via internet. The use of video cameras and personal computers is convenient for image uptake and storing. Tube cameras are problematic for uptake of pulsed images since the permanent erasing readout of the tube target requires a complicated data transfer process from the camera into the computer memory if the complete flashed image is to be saved.

With CCD cameras the transfer of pulsed images is much easier since the pixels store the incoming image over the frame time. This is the case even if it is flashed for only a few nanoseconds. At the end of the frame-time, the information of all pixels is shifted in a few microseconds into the memory portion of the sensor. From there they are serialized according to the video standard. Two kinds of CCD sensors are in use:

CCD frame transfer: half frames with pulsed exposure
CCD interline transfer: half and full frames with pulsed exposure

Full frame means that all pixels of the camera target are used. A half frame image contains only each second line of pixels of the camera target. With CCIR standard video systems 25 full frames or 50 half frames per second can be generated. Therefore the maximum image uptake is limited to 25 full frames/s or 50 half frames/s. Standard resolutions are

Full frame: H768xV512 (393KB) or H512xV512 (262KB)
Half frame: H768xV256 (196KB) or H512xV256 (131KB)

With full frame images, a maximum data stream of about 10MB/s can be generated and has to be stored on disk or tape. For digitally storing the images either standard or hardware specific formats are in use. Best quality is obtained with uncompressed full frame images.

Triggering the image uptake

The flow cytometric analysis is used to trigger the image uptake, i.e. the trigger problems are similar to problems of triggering a flow cytometric sorter. The runtime $T_d = l/v$ for making the decision and triggering imaging depends on the flow speed v

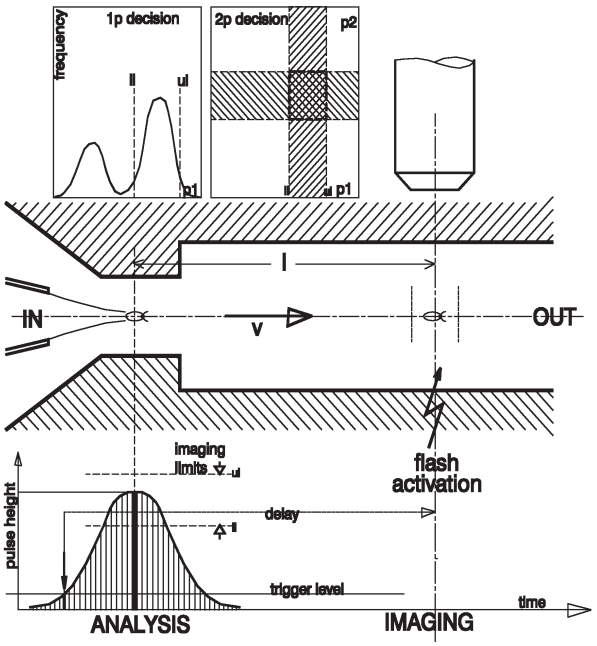

FIG. 3. – The principle of image triggering; v: moving speed of the objects, l: distance along the flow path between analysis and imaging, ll: lower level for the imaging decision on the parameter axis p_n, ul: upper level for the imaging decision

and the distance l of the measuring and imaging location in the transducer (Fig. 3). With $l = 1000\ \mu m$ and a speed of $v = 4 m/s$ $T_d = 250\ \mu s$. The controlling computer uses this time to make the required decisions. Critical, however, are the stability of triggering, the runtime defined by the flow conditions and the delay-time. The moment of flash activation for image uptake is determined by a delaytime which itself is triggered by the image decision device. If the diameter of the field of view of the imaging optics is $40\mu m$, the moving particles are only visible for $10\mu s$ in this field. If the nominal imaging point is in the center of the field of view and the trigger delay is fixed, an overall change of the mentioned parameters by $5\ \mu s$ means that the particle to be imaged is not yet in or has already left the field of view when the flash is triggered.

IMPLEMENTED FCI SYSTEMS

Macro Flow Planktometer

The Macro Flow Planktometer(MFP) was built within the Euromar project MAROPT (Fig. 4). It can be equipped with orifices between 0.8mm and 4mm diameter and measures Coulter volume and fluorescence and/or 90° scatter. Each parameter

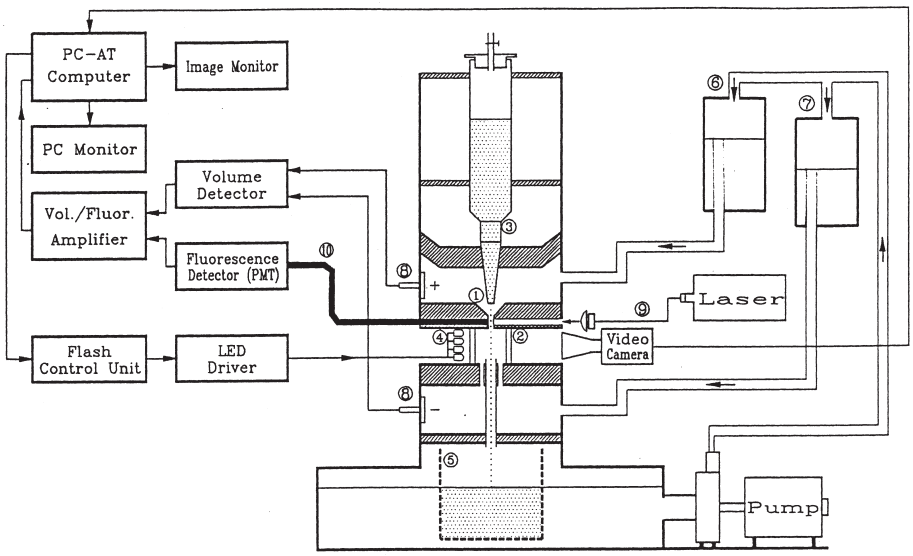

FIG. 4. – The Macro Flow Planktometer: 1 measuring orifice, 2 imaging field, 3 supply vessel, 4 LED unit, 5 collecting net, 6,7 sheath flow regulators, 8 electrodes, 9 laser input fibre, 10 signal out fibre

alone or combined with a second one can be used to trigger integrated imaging. The recirculating sheath fluid is pumped into the pressure regulators 6 and 7. The fluid levels in these vessels, which can be adjusted in height hydrostatically define the pressure conditions in the main measuring column. 6 is mainly responsible for the flow from the particle container 3 into the Coulter orifice 1. The particle flow is additionally controlled by the air-valve on top of the particle vessel. Regulator 7 controls among others the second sheath into the catcher tube, which collects the particles, which have passed the measuring orifice and the imaging field 2. For optical measurements a rectangular orifice with two transparent walls for introduction of the laser excitation is used. Fluorescence and scatter are taken rectangularly to the laser beam through a window in the orifice wall and conducted through a fiber into the PMT -detector box. The Coulter current is supplied by the electrodes 8. When the organisms have passed the analyzing orifice, the computer decides according to the imaging conditions to activate the flash control unit and the LED driver. The standard BW frame-transfer-video camera is continuously in

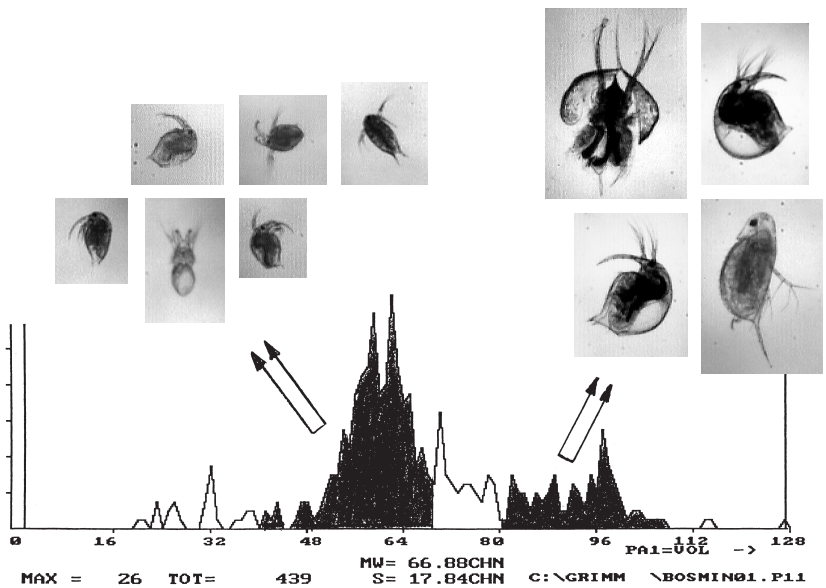

FIG. 5. – Application of the MFP: Images triggered from two different regions of a volume distribution curve of *Daphnia* and *Bosmina* are shown.

operation. When an image is flashed onto the target, the computer initiates its storage in the RAM memory and its display on the image monitor. A detailed description of the instrument is given in (Hüller *et al.,* 1994). The instrument is mainly suited for analyzing and imaging particles and organisms in the range of 100μm to about 4000 μm diameter. Analysis and image uptake is done by the same PC. The measuring rate is up to 500 1/sec. The maximum imaging rate without storing is 25 im/sec. When storing images on hard-disk, the imaging rate decreases to 1 to 2 im/sec. Figure 5 shows a measurement of fresh-water plankton containing mainly Bosmina and Daphnia. Two different regions of the volume distribution curve have been selected for imaging and a collection of images belonging to the two imaging regions are shown.

In a supplemental EUROMAR project, an electronically controlled electromechanical sorting module for the MFP was developed. A sorting MFP is used for benthos sorting at the AWI in Bremerhaven (Kachel *et al.,* 1995; Thiel *et al.,* 1995).

FCI module for the EUROPA system

Figure 6 illustrates the principle of the imaging module for the European Optical Plankton Analyzer which was developed within the scope of the MAST II EurOPA project. The EurOPA System analyzes only optical signals. The transducer consists of a cuvette with a square inner channel of about 50 mm length and 1 mm thickness. The imaging point 3 is 25 mm downstream from the analyzing point 2. From this long distance the runtime for the particles between the analyzing and imaging point is 10ms to 50ms depending on the flow velocity. As mentioned above this runtime has to be carefully stabilized by means of a well elaborated pressure- and hydrodynamic focusing system. This is necessary to minimize the number of missed particles in the imaging window. The realistic size range for imaging in this instrument is above 5 μm diameter. The imaging module is largely independent of the analyzing system: merely the trigger pulse is required for initiating the image uptake and the

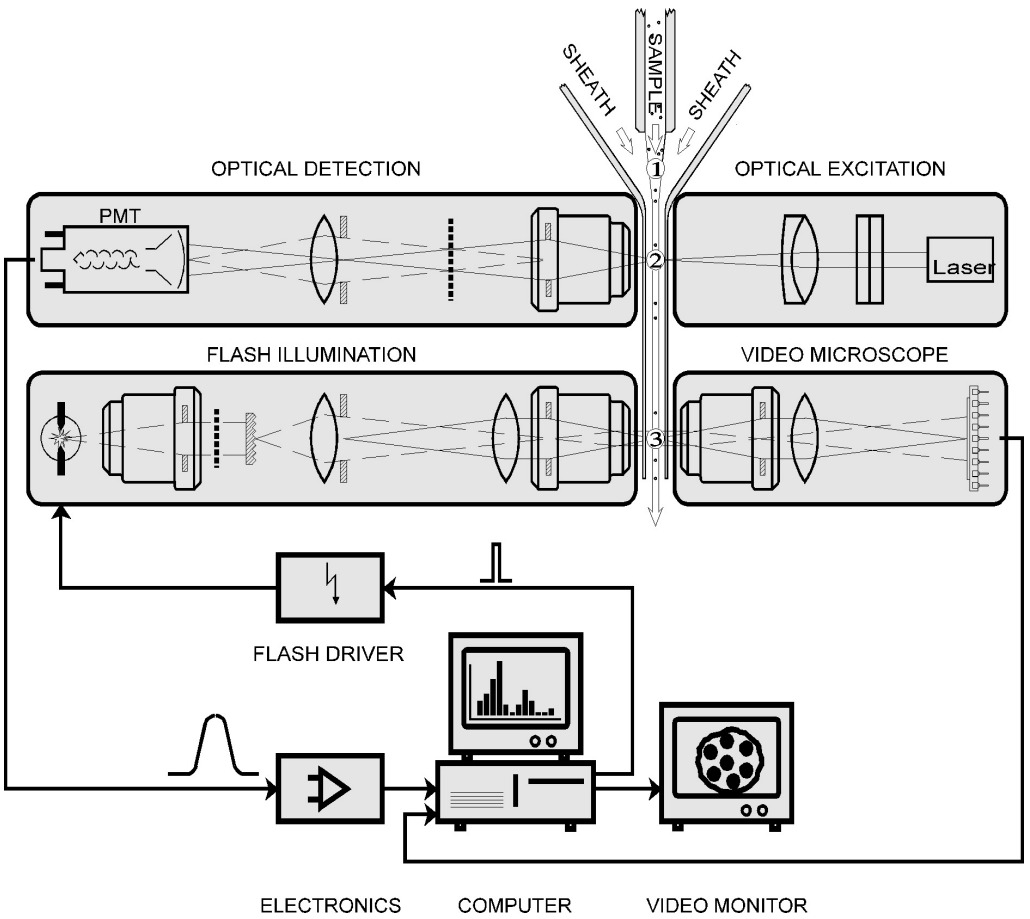

FIG. 6. – The principle of the EurOPA imaging module. 1: hydrodynamic focusing of the flow path, 2: Flow cytometric analysis point, 3: imaging point.

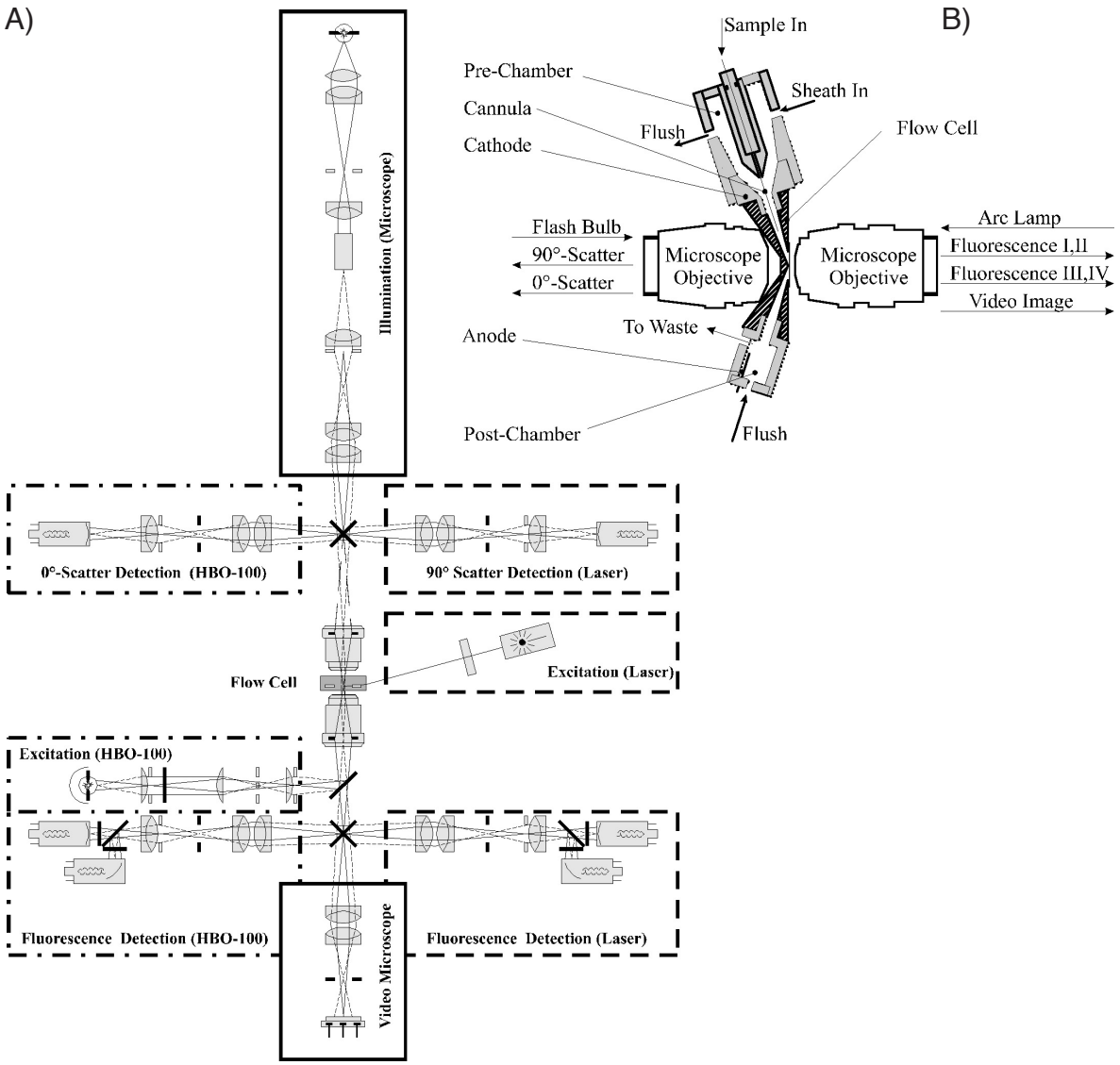

FIG. 7. – (a) Schematic block-drawing of the optical system of the FluvoVI flow cytometer. (b) The sensing head with the flow cell and the signal paths, the exciting laser beam is rectangular to the plane of drawing

optics have to be mounted on the tranducer. Storing the images is done in a separate imaging PC. A detailed description of the system is given in (Wietzorrek *et al.,* 1994; Anonymous, 1995). An imaging-in-flow system for marine applications was also developed by (Sieracki *et al.,* 1998). The image uptake can be triggered by a fluorescence detector. Since the size of measured objects has to be calculated from the video images, its achievable analysis-throughput is restricted.

Fluvo VI Flow Cytometer

Fluvo VI is a recently developed universal flow cytometer with means for Coulter sizing, four fluorescence channels, forward and side scatter and flow cytometric imaging (bright field illumination and phase contrast imaging). The instrument is presently in use for experimental cell research at the institute for experimental surgery at the Ludwig-Maximilian-University in Munich. The instrument is equipped with 2 excitation systems, a HBO 100 arc lamp and an Argon ion laser, which can be used simultaneously. The pulsed light source for FCI is a Nanolite flaslamp of 18 ns light pulse duration. Analysis and Imaging occur in the same orifice with very short delay.

Figure 7a shows schematically the optical arrangement of the system and Figure 7b the quartz glass made flow cell with the in- and outgoing sig-

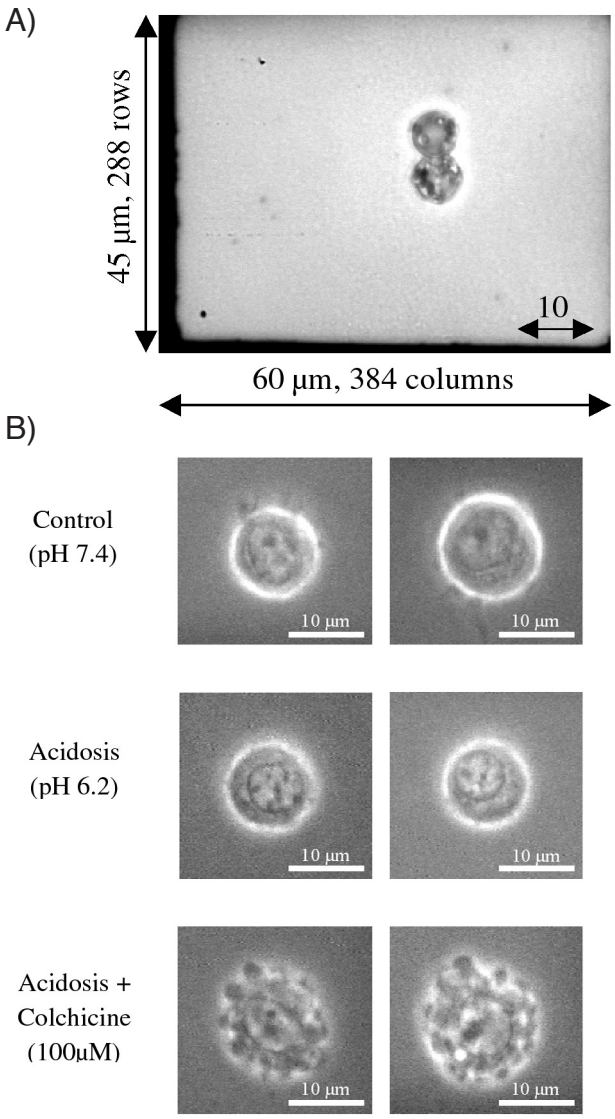

FIG. 8. – Fluvo VI flow images: a): latex spheres, imaged in bright field mode; b): glioma cells, imaged in phase contrast mode Acidosis leads to condensation of cellular chromatine Colchicine causes blebbing of the cell membranes

nals. The data acquisition system is capable of measuring up to 8 parameters. Each of the parameters, or a combination of two, can be used for image triggering. Image uptake is triggered by a computer controlled hardware device due to the very short delay time between analysis and imaging. More details of the system are shown in (Wietzorrek, 1999; Wietzorrek *et al.,* 1999). Two application examples are given in Figure 8. Several structures can be recognized on the pair of 6.25 μm diameter latex particles of Figure 8a. The distinguished resolution power of phase contrast imaging in flow is demonstrated by the images of glioma cells (Fig. 8b). Here, the inner cell structure can be recognized in detail. But one has to bear in mind that such phase images may differ from usual absorption images.

FUTURE DEVELOPMENTS

The main problem of flow cytometric imaging is the motion of the objects. Improvements in resolution cannot solely be obtained by improved cameras or optics or high resolution targets. The first problem to be solved is to further stabilize the local position of the flow path. With high resolution targets the pixels are of smaller size, i.e. with identical flow speed the illumination time has to be reduced in order to avoid unsharp images by motional displacement. Shorter illumination time with smaller sized pixels, however, means that the available light on each pixel is twofold reduced. For higher resolved low noise images the sensitivity, or better the signal to noise ratio of the target has to be considerably increased e.g. by cooling. Cells and organisms are of three-dimensional structure. It is therefore highly

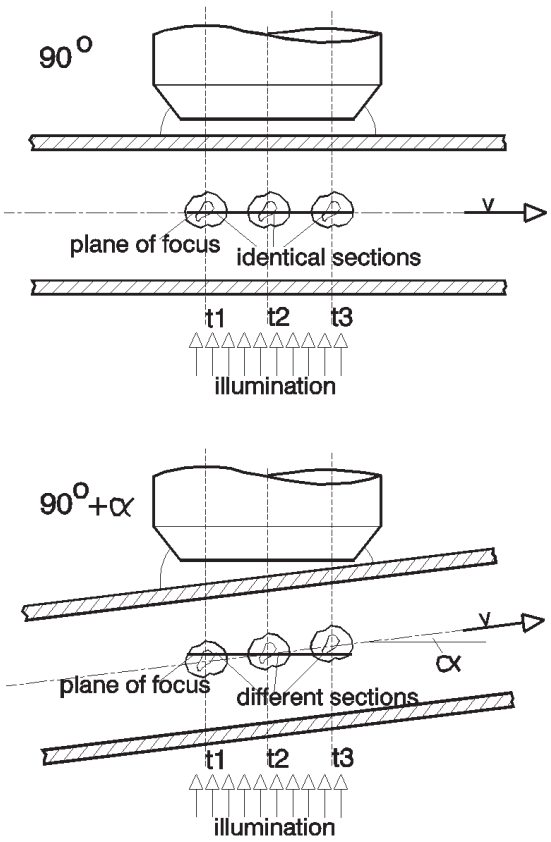

FIG. 9. – 3-D imaging in flow. By multiflash-imaging on an inclined flow path several sections of the same object are imaged.

interesting to get more than one section image through the images objects. Figure 9 shows a simple principle already discussed earlier (Kachel *et al*., 1980) how several sections through the same flowing object can be obtained. By inclining the direction of the flow path to the plane of focus by a small angle and imaging the objects several times a series of sections through a particle can be obtained. Flashes applied at the short distant times t1-t3 image the particles with the plane of focus at three different sections. With a properly designed transducer several sections can be taken by the same camera target, stored in a single image and the 3D structures can be composed by computer programs.

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