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TECHNICAL NOTES

Signal Processing in Slit-Scan Flow Cytometry of Cell Conjugates

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The design and implementation of a real-time signal processing system for slit-scan flow cytometry is described. The system is used to measure the separate scatter and fluorescence peak heights of 2 adherent cells. Preliminary measurements of changes in the membrane potential induced by interactions between natural killer (NK) cells and their target cells are presented. © 1993 Wiley-Liss, Inc.

Key terms: Adherent cells, natural killer cells, cytotoxic cells

In order to measure physical aspects of cell-cell interaction using a flow cytometer, it is necessary to measure the individual scatter and/or fluorescence signals of adherent cells (conjugates). In principle these individual signals can be obtained by using slit-scan illumination (3,10). If the 2 cells that form the conjugate have similar sizes, the hydrodynamic forces in the funnel of a typical flow cell will orient the cells along the direction of flow. Therefore a slit-scan illumination can resolve the individual cells. In previous work, the total slit-scan profile was measured using a transient recorder and was analyzed with a computer afterwards (8,9). For the particular problem of conjugated cells, one is only interested in the peak heights of the signals from individual cells.

In this report we describe a simple analog signal processing system for measuring the 2 peak heights of a slit-scan signal from 2 adherent cells. The timing of this system is based upon one input signal that always shows 2 peaks when a conjugate passes the slit, e.g., forward light scatter, and the first derivative of that signal. The zero crossing points of the first derivative indicate the maxima and minima in the signal and can be used to determine the contact region (minimum signal). The principle of taking the derivative of an input signal was used by van Oven et al. to identify the centromere position in chromosomes by the determination of the so-called pulse dip index (5). They did not measure the separate peak heights of the signal, but they used the derivative to calculate a new parameter, the ratio of the profile up to the first local minimum and the total area of the profile.

Our signal processing system has a modular construction which can be used for different purposes by changing the logic circuit. This logic circuit can be easily changed because it is designed using an erasable programmable logic device (EPLD). Two peak detectors are used for every parameter, which requires double peak evaluation. The processing time is dependent on the length of the signal, and the double peak evaluation causes no extra delay.

Measurements on the fluorescence changes of a potential sensitive dye, induced by interactions between natural killer (NK) cells and their target cells (K562), are presented as an illustration of the possibilities of this signal processing system.

MATERIALS AND METHODS

Instrumentation

The signal processing system was implemented in a self-made flow cytometer described elsewhere (1). Light of an 100 mW argon ion laser (model 5500 AWC,

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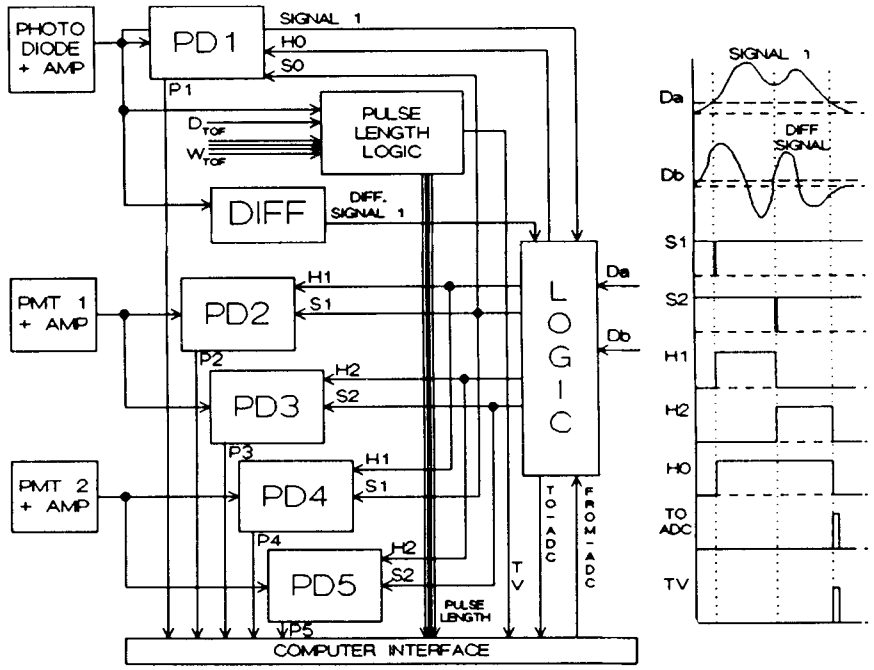


FIG. 1. Schematic drawing of the signal processing system and the corresponding timing diagram. PD = peak detector; DIFF = differentiator; PMT = photomultiplier tube; AMP = amplifier. S0 and H0 are the sample and not-hold signals for the total signal, S1 (= S0) and H1 are the sample and not-hold signals for the first peak, and S2 and H2 are the sample and not-hold signals for the second peak. Da is the lower discrimination level for the first input signal. Db is the lower discrimination level for the derivative of that signal. D_{TOF} is the discrimination level for pulse length determination. The signal TV is generated if pulse length is within the discrimination window W_{TOF}. P1 to P5 are the peak values of signals 1–5.

Ion Laser Technology, Salt Lake City, UT), with λ = 488 nm, polarized parallel to the direction of flow, was focused to an elliptic spot of approximately 4.9 μm in the direction of flow and 100 μm in the direction orthogonal to flow using a cylindrical lens (focal length 80 mm) and a Leitz 32/0,40 objective. A square flow cell (Hellma Gmbh, Germany) with a conical part of 4 mm and a flow channel of 1 mm was used. Three signals were measured: forward light scatter, green fluorescence, and orange fluorescence.

A schematic drawing of the system used and the corresponding timing diagram are given in Figure 1. A signal that always shows 2 peaks when a conjugate passes the slit is used as input signal 1. In our setup this is the forward light scatter signal. This signal is used as an input signal for a peak detector (PD1), a differentiator, and a logic circuit. The differentiated signal is also used as input signal for the logic circuit.

The self-made peak detectors, shown in Figure 1, consist of a sample and hold circuit (HA5320), and a comparator (LM319); the electronic scheme is given in Figure 2. The basic principle of this peak detector is that after it has been activated by a start sampling pulse, S, the sample and hold circuit is continuously triggered as long as the input signal is higher than the output signal and as long as the not-hold signal, H, is active. The dynamic range of the pulses than can be measured with these PDs is 10 mV to 10 V. The derivative is obtained by a network of an inverter and an inverting differentiator, represented in Figure 3.

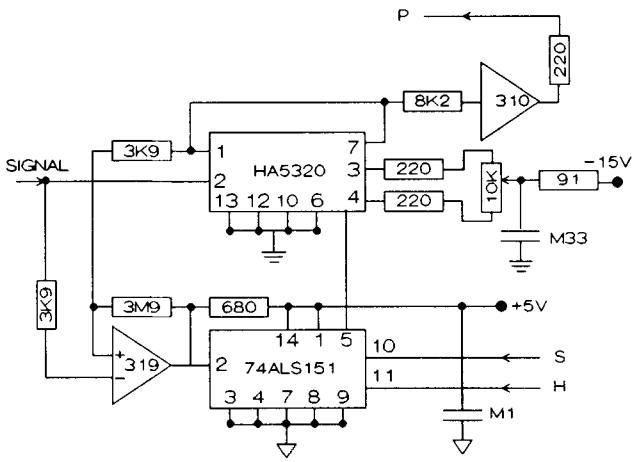


FIG. 2. Electronic scheme of the peak detector (PD) shown in Figure 1. S is the start sampling pulse, H is the not-hold signal, and P is the peak value.

When signal 1 has exceeded Da, and the differentiated signal has exceeded Db, the start sampling signals S0 and S1, and the initialization of the not-hold signals H0 and H1, are generated by the logic circuit. If the derivative of the input signal 1 exceeds Db for the sec-

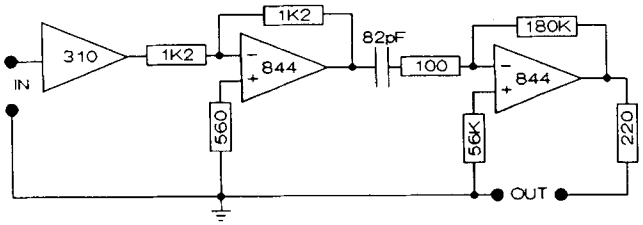


FIG. 3. Electronic scheme of the differentiator shown in Figure 1.

ond time (in case of a second cell), or if signal 1 gets below Da again (in case of a single cell), and not-hold signal H1 is ended. If signal 1 has not ended yet, this event also generates the sampling start signal S2, and it initializes the not-hold signal H2 for detection of the second peak in the signal. All the not-hold signals H0, H1, and H2 are ended if the input signal gets below Da again. This also generates the data valid signal for the computer, TO-ADC. If more than 2 cells are conjugated, only the first 2 cells are measured. The measurement cycle is enabled by a signal from the computer, FROM-ADC.

In our setup PD1, therefore, detects and stretches the highest peak in the total forward light scatter signal. PD 2 and PD 4 are set to detect the height of the first peak of the amplified outputs of photomultiplier tubes (PMT) 1 and 2, respectively. PD 3 and PD 5 are set to detect the height of the second peak of the respective signals. The first input signal is also used to determine the pulse length or time of flight (TOF) by counting the clock pulses during the time that the signal is higher than the adjustable level D_{TOF}. Using 3 input signals, there are 6 output signals for the interface to the computer, 5 analog signals (peak heights of the forward light scatter and of the 4 fluorescence signals), and 1 digital signal (time of flight). A separate window discriminator is used to generate the signal TV if the time of flight is within the preset window W_{TOF}. The signal TV can also be used as a TO-ADC signal for the computer; in that case only cells that have a time of flight within a certain interval will be measured.

The sample and hold signals for the PDs and the handshake signals for the interface with the computer are generated by the logic circuit represented in Figure 4. It consists of 4 analog window discriminators (electronic schemes obtainable upon request) and an erasable programmable logic device (EPLD, Intel D5C090) with latched inputs and outputs. Only 2 of the 4 window discriminators are used: The first one is used for signal 1 and the second one for the differentiated signal.

The EPLD is programmed to generate the sample and to hold signals for the 5 peak detectors and the handshake signals for the computer interface. It is configured to contain 3 separate state machines. The EPLD uses a clock with a period of 100 ns. At a clock pulse, the output state of a state machine can be al-

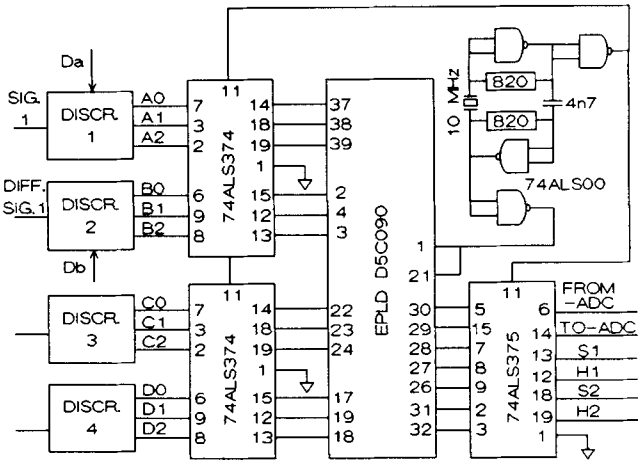


FIG. 4. Electronic scheme of the logic circuit shown in Figure 1. A1, B1, C1, and D1 are signals that are generated when the signal exceeds the lower level of the discriminator. A2, B2, C2, and D2 are signals that are generated when the signal exceeds the upper level of the discriminator. A0, B0, C0, and D0 are signals that are generated when the signal exceeds half of the lower level of the discriminator (used for generating the sample pulses). Da and Db are the adjustable lower levels of the first 2 discriminators.

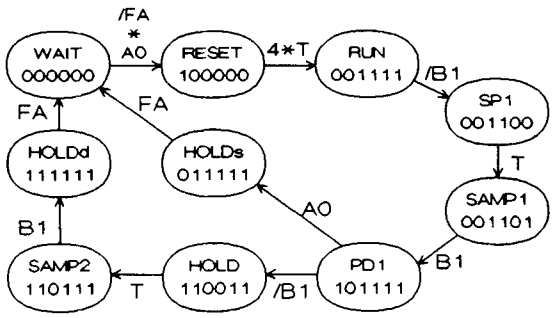


FIG. 5. Machine state diagram of the part of the EPLD used for double peak detection. T is the clock period (100 ns); FA is the signal FROM-ADC. A0 is negative if the input signal exceeds Da, and B1 is a negative if the derivative of the input signal exceeds Db ("/" means not). Four of the 6 bits shown are output lines: The first 2 (from left to right) are used for discrimination between the different internal states, the third is H2, the fourth is S2, the fifth is H1, and the sixth is S1.

tered conditionally on the input signals (using IF-THEN statements, CASE statements, or truth tables) or unconditionally. The machine state diagram of the machine responsible for timing of the PDs 2–4 is given in Figure 5. Four of the 6 bits of this machine state are used as output lines for generating the timing signals of the PDs: The first 2 bits (from left to right) are only used for discrimination of the different states. The third bit is used for H2, the fourth bit for S2, the fifth bit is H1, and the sixth bit for S1. The signals H0 and S0 are generated by another similar machine (not shown here).

The time scale in the timing diagram of Figure 1 is

dependent on cell size, spot size, and stream velocity. The measurement of 2 peak heights in one signal causes no extra delay compared to the measurement of a single cell with the same pulse width. In our measurements the time scale in Figure 1 is about 20–30 μs.

Measurements

The signal processing system was used to investigate the membrane potential changes of K562-cells and natural killer (NK) cells when being conjugated. Cells were maintained in suspension cultures as described elsewhere (6). The membrane potential was monitoring using the fluorescent dye $DiBAC_4$ (3) (Molecular Probes, Eugene, OR; concentration 200 nM), which emits at 520 nm. The concentration of the dye inside the cell is dependent on the membrane potential. There is an inverse relationship between the fluorescence of this dye and the membrane potential: If the membrane potential decreases (depolarization of the cell membrane), more dye enters the cell and the fluorescence increases, and vice versa. Because of this mechanism, both cell types will be stained with DiBAC. In order to identify the different populations of single cells and conjugates, an extra parameter was used: The NK cells were loaded with the vital fluorescent dye TR18, which binds to the membrane (emission of this dye is around 580 nm) (4). NK Cells were incubated with TR18 over 1 h (37°C) and were washed 2 times. The membrane potential was monitored as a function of time. NK and K562 were centrifuged together over 1 min at 200 g and then resuspended. DiBAC was added to the suspension 3 min before each measurement in order to achieve equilibrium. In between the measurements, the cells were kept at 37°C.

In all measurements 16384 events were measured in listmode: The first 4096 events were not gated, and the last 12288 were only conjugates (gated using the time of flight signal). One measurement took about 5 min. The stream velocity in the funnel was about 1 m/s. The throughput rate was about 200 cells/s.

Software

The measurements were analyzed using a self-made analysis program. One measuring event contains information about the total signal (forward light scattering and time of flight) and information about the individual cells that contribute to that signal (fluorescence intensities). The information about the first cell in any event is contained in parameters 2 and 4, and the information about the second cell is contained in parameters 3 and 5.

If we denote one cell type with A and the other with B, one can discriminate at least 6 populations: A, B, AA, BB, AB, and BA. The ratio between the number of ABs and the number of BAs is dependent on orientation preference. The information about the individual cells is in different parameters for these 2 populations. To obtain good statistical results, we implemented in our analysis program the possibility of adding these 2 populations: After identification of the 2 populations, using gating analysis, we transformed the BA population in AB by swapping parameter 2 with parameter 3, and parameter 4 with parameter 5, for all measuring events belonging to population BA.

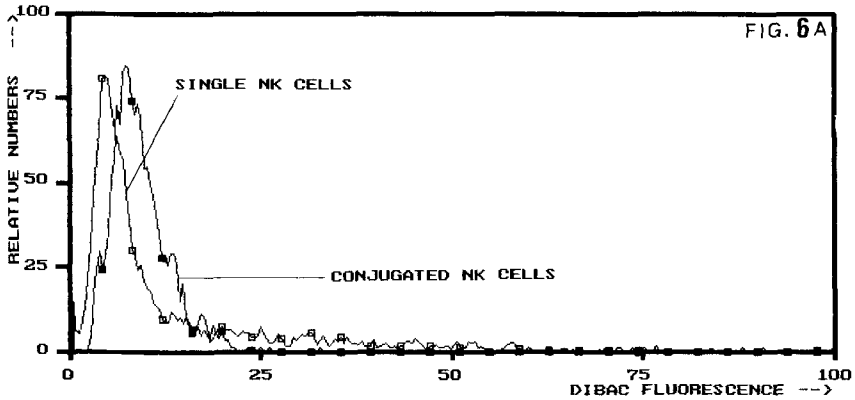
RESULTS AND DISCUSSION

Using the data acquisition system for measuring double peak heights, we investigated the membrane potentials of NK and K562 cells that are conjugated, using the potential-sensitive dye DiBAC. Figures 6 and 7 show the DiBAC fluorescence histograms of K562 and NK cells after incubation times of 0 and 20 min, respectively. All populations were identified using the gating analysis program. In all histograms the NK-K562 conjugate populations were added as explained above. In the histograms 6A and 7A, all single and conjugated NK cells are shown. In histograms 6B and 7B, all single and conjugated K562 cells are shown. After 20 min a part of the conjugated K562 cells show a higher DiBAC signal (depolarization of the cell membrane). Most of these cells did not stain with propidium iodide. These results indicate that the lytically active NK cells induce a depolarization of the target cells before the target cells are completely lysed.

The signal processing system described here can only be used for measuring 2 adherent cells. Correlation errors can only occur if conjugates consisting of more than 2 cells are measured. This will not create problems if the cells form chains. Then only the first 2 cells are measured, and these events can be discriminated using the pulse length measurement. However, if cells form clumps, signals of different cells can be attributed to one single cell, which can give false interpretations.

The noise level that can be tolerated in the signal that is used for timing of the double peak detection is frequency dependent. False triggers caused by rapid variations in the signal can be prevented by choosing an appropriate time constant for the differentiator. False triggers caused by slow variations in the timing signal or by noise in the differentiated signal can be eliminated by adjusting the level DB of the window discriminator for the differentiated signal (see Figure 1). The level DA of the window discriminator that is used for the timing signal should always be set lower than the minimum in the timing signal, representing the contact region of the adherent cells. If not, a conjugate will be regarded as 2 separate cells. The noise should, of course, be smaller than the difference between the minimum and smallest maximum in the signal.

In general, one can adjust the spot size, flow velocity, and bandwidth of the differentiator to the signal resolution that is needed for a specific application. The minimum pulse width that can be double peak detected using the differentiator shown in Figure 3 is about 5 μs. The maximum pulse width that can be detected with the same dimensions of the differentiator is at least 100 μs. The minimum pulse width is sufficiently



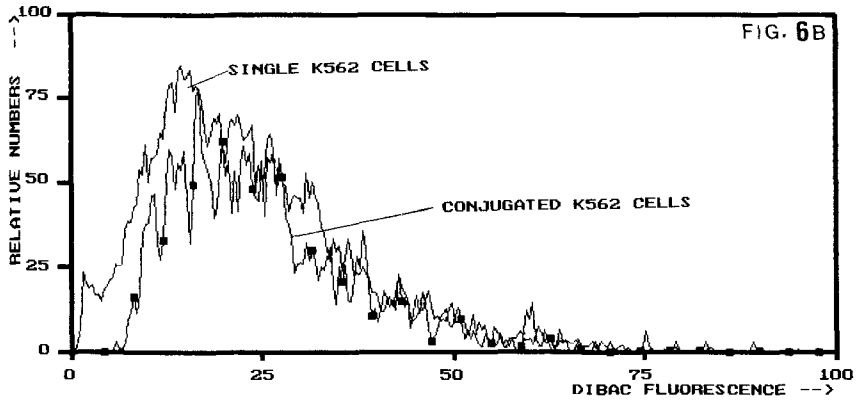
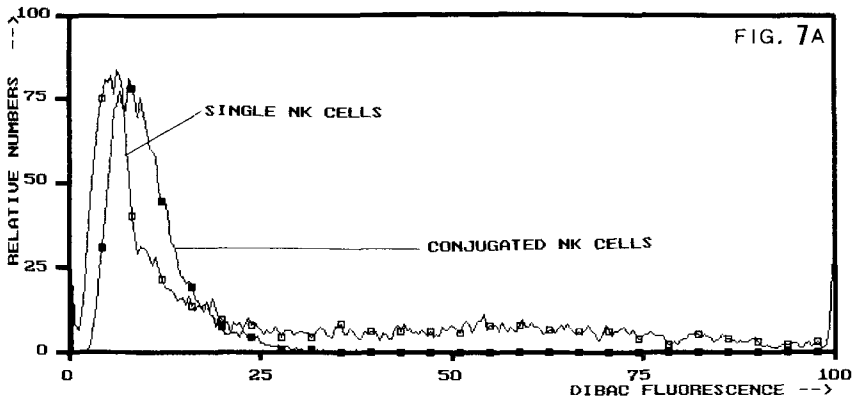


FIG. 6. Histograms of DiBAC fluorescence intensities of single and conjugated NK and K562 cells after 0 min. **A:** Histograms of single and conjugated NK cells. **B:** Histograms of single and conjugated K562 cells.



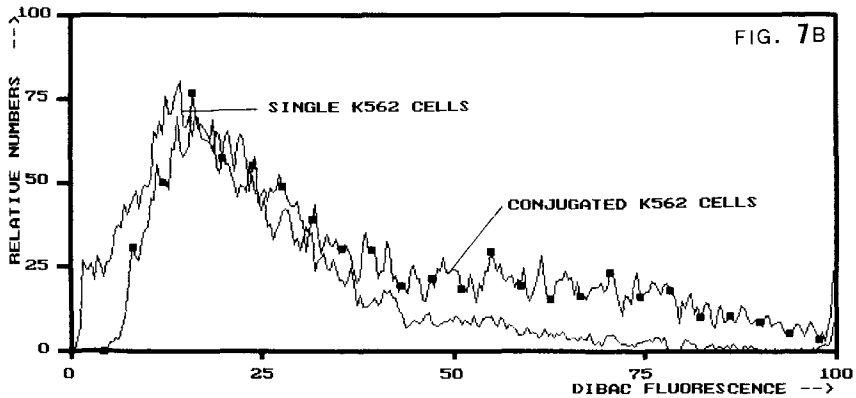


FIG. 7. Histograms of DiBAC fluorescence intensities of single and conjugated NK and K562 cells after 20 min. **A:** Histograms of single and conjugated NK cells. **B:** Histograms of single and conjugated K562 cells.

small for discriminating the separate peaks in slit-scan signals of conjugates of cells that are about 10–15 μm in diameter, using a laser spot size of 4.9 μm and a flow velocity of 1 m/s.

When measuring a double peak, there is no way of telling whether the cells actually stick together or if the double peak is caused by coincidence. Therefore the flow rate should be appropriately low to reduce the number of mistakes caused by coincidence. The coincidence probability P for 2 cells to arrive at the measuring point within the dead time t can be calculated using a Poisson distribution: $P = 1 - e^{-\beta \cdot t}$, where β is the number of cells measured per second. In our measurements we used a flow rate of 200 cells/s; with a dead time of 25 μs (measuring time) the coincidence probability is 0.5%.

The signal processing system described here is a simple and efficient way to get direct information out of slit-scan flow cytometry signals. It can also have useful applications in the study of the cell fusion process. Studies of the diffusion of membrane-bound fluorescent labels on fused cells (2), now performed using microscopic techniques, can be done on a much larger scale in a flow cytometer. Another possible application might be the reduction of the number of mistakes caused by coincidence in high-speed flow cytometers.

CONCLUSIONS

In this report we described a signal processing system for real-time measurements of peak heights in a slit-scan doublet signal. The use of EPLDs and modular construction make it very easy to implement a signal processing system dedicated for a particular application. Using this signal processing system, we measured the separate membrane potential changes of conjugated cells, using a potential sensitive dye. The preliminary results, shown here, indicate that these changes can be measured clearly using slit-scan illumination and the signal processing system described in this report. A more extensive study about membrane potential changes in NK-target cell conjugates has been submitted for publication (7).

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