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Laser-based blood cell measurements for the biological reconstruction of ionizing radiation doses using a novel technique for cytometry/biochemical reaction analysis.

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ABSTRACT

A novel biodosimetry method based on the red blood cells analysis by means of laser flow cytometry and a specially designed monoclonal antibodies is used for reconstructing ionizing radiation doses for Chernobyl Accident victims. In addition to analysis of the patient's blood samples, we are evaluating both spectroscopy procedures and laser-based flow techniques. It was shown that resonance energy transfer processes can take place among the two different dyes links to the spherical cell surface. This can affect the accuracy of rare mutant cell scoring. Also a new technique is described to provide conventional flow cytometric analysis and kinetic measurements of the elementary stages of biochemical reactions are described.

2. INTRODUCTION

The Glycophorin-A (GPA) assay [1-3] uses immunofluorescence labeling and flow cytometric analysis to enumerate the frequency of red blood cells lacking expression of one allelic form of the cell-surface sialoglycoprotein, GPA, presumably as a result of allele-loss mutations at the GPA locus in erythroid precursor cells. The GPA gene is located on human chromosome 4 and is present in the population as two common alleles at approximately equal frequencies, so that ~50% of the population is MN heterozygous. The proteins coded by these two alleles carry the M and N blood group antigens and differ by only two amino acids out of 131. The measurements with this assay enumerate the frequency of NØ variant cells, i.e., cells that have lost expression of the M allele but express the N allele normally. The number of scored variant cells corresponds to the ionizing irradiation dose for the individual, and variant cells appear to accumulate with doses received throughout the patient's lifetime [4,5].

We have previously reported [6] that these principles and assays are now being used for an International Project related to the Chernobyl accident to reconstruct the doses received by accident victims and persons who participated in the clean-up works after the accident. By looking at different patients, we are trying not only to reconstruct their doses, but to depict the differences and similarities among different nuclear accidents. For example, an important consequence of the similarity seen between the Hiroshima A-bomb survivors and the Chernobyl studies is the conclusion that the mutagenic effect, i.e. the similar induction of GPA mutations per unit dose, of irradiation on human bone marrow does not depend strongly upon the dose rate of exposure. The existence of a measurable dose-response and the long-term persistence of the glycophorin A effects in radiation exposed individuals indicates that the glycophorin A based somatic mutation assay could serve as a cumulative biodosimeter, e.g., it might be used to monitor individuals who work in occupations with a risk for chronic exposure to ionizing radiation. This assay also might serve as a cancer risk estimator for each individual.

3. CELL PREPARATION FOR GPA ANALYSIS BY FLOW CYTOMETRY

For scoring the of a number of mutant cells with the GPA assay, erythrocytes are separated from the peripheral blood of the patient. Biochemical procedures [1-3] using a formaldehyde solution, Isolite and staining buffer solutions cause the erythrocytes to become spherically shaped. This spherical shape makes all of the cell surface more accessible for monoclonal antibody binding, and also results in uniform light scattering intensities for the cells in the flow cytometer. Two types of the specially designed monoclonal antibodies, recognizing specifically the M or N type of glycophorin molecules, are simultaneously linked to the erythrocyte's spherically shaped surface. Each of the antibodies is labeled with fluorescent dye molecules (directly or via biotin/streptavidin). R-phycoerythrin (PE) and Fluorescein (F) are the fluorescent labels used in the assay. Green fluorescence is emitted from fluoresceinated anti-GPA-N antibody and orange fluorescence is emitted from phycoerythrin-conjugated anti-GPA-M antibody.

Fluorescence and absorption spectra of Fluoresceine and R-Phycoerythrin

Fig. 1

Since there is overlap between the Fluorescein emission and R-Phycoerythrin absorption bands, there is the potential for spectral interaction between the dyes when they are linked to the cell surface via monoclonal antibodies. If the position and orientation of the dyes molecules on the spheroidal erythrocyte surface are closed enough, resonance energy transfer can occur.

Fig. 2

This simplified diagram shows the erythrocyte membrane, monoclonal antibodies, and fluorescent dyes. From the electron microscopy data [7], it is possible to estimate approximately the mean distances among dye molecules and the approximate energy transfer efficiency among these dye molecules

3.2. ENERGY TRANSFER AND OTHER SPECTROSCOPIC PHENOMENON RELATED TO GPA-ANALYSIS

Each of the erythrocyte surfaces contains about 5×10^5 glycophorin molecules. Erythrocyte spheres have a diameter of about $5-7 \mu\text{m}$. The mean square of the cell surface where one can find 1 glycophorin molecule with the assumption of homogeneous distribution of glycophorins on the sphere:

$$S = \frac{4\pi R^2}{\langle c \rangle} \cong 630 \text{ nm}^2 / \text{molecule};$$

$$c \approx 5 \cdot 10^5;$$

About 30% of the GPA molecule (131 amino acids) is outside of the membrane yielding an external fragment length of ~70-80 Å. Monoclonals used are the IgG components. With the fluorescein label, their length is ~250 Å, assuming that the fluorescein molecule size is about 5x5 Å. The R-Phycoerythrin-streptavidin-MoAb conjugate length is estimated to be ~450-500 Å. From these geometrical parameters it is possible to determine that the mean distance among neighbor GPA molecules on the sphere surface, $R \sim 190-200$ Å.

Fig. 3

By integrating over the Fluorescein emission and R-Pe absorption spectral bands overlapping range and assuming that orientation factor $\sim 2/3$, the Forster radius value can be calculated as follows :

$$R_0^6 = \frac{9\chi^2c^4}{8\pi N_b} \int F_s(\omega)F_b(\omega) \frac{d\omega}{\omega^4}; \quad R_0 \approx 90 \text{ Å}; \quad R = 2.1 R_0;$$

N_b – acceptor's concentration;

$F_d(\omega)$ – donor's emission spectrum;

$F_a(\omega)$ – acceptor's absorption spectrum;

The related energy transfer efficiency is:

$$\epsilon = \frac{R_0^6}{R_0^6 + R^6} \cong 0.03;$$

One can conclude that the energy transfer among the dye molecules bounded on the erythrocyte surface can to change the relationship of the fluorescence intensities when the two dyes are excited by the same laser. In the case of flow cytometry regime these two fluorescence signals are recorded using a two-dimensional scale:

$$I^F(FL) \{ I^A(R - Pe) \};$$

The next step in the GPA assay is the scoring of the number of variant cells (per million normal cells) which are labeled with only one dye (F). This number corresponds with the radiation dose. The scoring of the rare mutant cells is carried out using "windows" whose size and position in the two-dimensional intensity scale is selected empirically [3].

Fig. 4

The position, height, and bounds of the rare mutant cells "windows" are selected to correspond to cells having the presence or absence of one of the dyes on the erythrocyte surface. If one of the dyes is totally absent (variant cells), no energy transfer can occur. Energy transfer is possible for "normal" MN cells ("main cells" area) and for cells with a partial loss of GPA (M) (windows height). While the number of variant cells is very small (typically a few variant cells per million erythrocytes), the accuracy of the selection of the windows positions and sizes can affect the accuracy of the dose reconstruction. Other

spectroscopic features such as multiple dyes bound to each monoclonal antibody molecule, or inhomogeneity in the distribution of glycoproteins on the cell surface, may also be important to evaluate for improvement of the biosimetry. Moreover, one of the general spectroscopic problems related to flow cytometry, the resonance energy transfer among randomly oriented donor-acceptor pairs linked to a spherical surface, has not yet been completely solved.

3.3. A NOVEL TECHNIQUE FOR BIOCHEMICAL REACTION ANALYSIS

Methods for rapid chemical reaction kinetics analysis (e.g. stopped-flow, T-jump, etc.[9]) utilize rapid reagents mixing for the primary initiation of reactions provided followed by recording of the optical parameters in the reaction mixture microvolume. For these microvolumes, it is assumed that the reaction stages are synchronized for all reagents. Rapid time-resolved spectroscopy (particularly, intrinsic enzyme chromophore fluorescence lifetime measurements) for different elementary stages of biochemical reactions has been used recently for real-time studies of enzymatic reaction cycles [10]. Our new approach is to use the two-component flow in a flow cytometer nozzle for the rapid mixing of two reagents at the tip of the needle. Figure 5 shows a schematic view of flow in a nozzle with dimensions given in micrometers (μm). In normal flow cytometry, a sheath fluid (channel 1) is used to hydrodynamically focus a sample stream (channel 2) [11]. It is possible to use channels 1 and 2 to mix biochemical reagents (e.g. enzyme and substrate) in a time interval of about 10-20 μs , which is 1-2 orders of magnitude faster than in a conventional stop-flow apparatus (2-10ms)[9]. Our attempts to do this showed that the pre-mixed flow then continues to maintain laminary flow for distances of 10-15 cm from the nozzle tip.

Fig. 5

With this approach, vertical movement of the optical excitation/secondary emission detection system along the stream from the nozzle tip can be used to monitor the time-course of the different elementary stages of biochemical processes in the synchronized microvolumes of reaction mixture. If the excitation/detection's system position is fixed, and the focussing buffer solution and cell suspension are used in the channels, then the system can work as a conventional flow cytometer. Such a facility was designed by us and is called the "cytometer-microreactor" system.

3.4. LASER FLOW CYTOMETER-MICROREACTOR DESIGN. PARAMETERS. AND FEATURES

The cytometer-microreactor facility shown in Figure 6 consists of the following components: (1) an argon ion laser (cw, 1W, 488nm); (2) a Nd:YAG laser with a second harmonic generator driving a R6G tunable dye laser with a second harmonic generator (1-1000 pps, 20 ps, 2.5 kW/pulse); (3) an optical system for detecting secondary emission (nanosecond photomultipliers, interference filters, a 6 gigahertz (HHZ) ADC, and a CCD-camera); (4) a PC-386 computer; (5) a precision mechanical system for nozzle position adjustment; (6) and a hydropneumatic block for pressure stabilization reagents delivery.

Fig. 6

Careful evaluation of some of the basic parameters and characteristics of the system was required to predict the potential of this facility for both the flow cytometry and reaction analysis regimes. The nozzle sizes and the volume where the reagents are first mixed is shown in figure 5. For a flow speed of ~ 10 m/s, the primary mixing occurs in a time period of $\sim 150\mu\text{s}$. Two limiting cases are possible:

- a) total mixing of the reagents at the nozzle tip; or
- b) a coaxial two-component stream coming out of the tip that maintains laminary flow for a length of $\sim 10\text{-}15\text{cm}$.

The reaction takes place in the region of bilateral radial diffusion of the components, and this region expands with time at the different points along the stream. Vertical stabilization of the stream and the careful optical adjustments along the stream must be used to ensure identical conditions for optical excitation and light detection to measure the time-course of the reaction within the region of diffusion. The accuracy of the vertical adjustment of the stream is predicted by the condition that the maximal deflection from the vertical and from the excitation beam axis must not be more then $\sim 170\mu\text{km}$. Thus, at the maximal distance from the tip where the stream is still laminary (10-15cm) the angular deflection from the vertical may not be more then 3 degrees. The mechanical positioners used for the nozzle and laser mirror provide an accuracy of angular deflection of 0.3 degrees.

To evaluate the changes caused by the chemical reaction development in the two-component stream, the corresponding diffusion equations were solved. The inner stream radius was assumed to be $\sim 75\mu\text{km}$, and the external stream radius was $\sim 400\mu\text{km}$. If the diffusion coefficient, D , is assumed to be the same along the stream the equations will be:

$$\frac{\partial n}{\partial t} = (\nabla^2 n)D; \quad \text{for the diffusion from the internal stream;}$$

$$n(r,0) = \theta(r-a)n_0 -$$

$$n(r,0) = [1 - \theta(r-a)]n_0; \quad \text{for the diffusion from external stream.}$$

The boundary conditions at the external bound are:

$$\frac{\partial n(r,t)}{\partial r} \Big|_{r=R} = 0;$$

The solution was found in the form of the Bessel functions sum and expressed as:

$$n(r,t) = \frac{2n_0a}{R} \sum_{m=1}^{\infty} \frac{J_1\left(\frac{a_m a}{R}\right) J_0\left(\frac{a_m r}{R}\right)}{J_0^2(a_m) a_m} e^{-\frac{D a_m^2 t}{R^2}} + n_0 \frac{a^2}{R^2}; \quad (\text{"internal" case})$$

Or:

$$n(r,t) = -\frac{2n_0a}{R} \sum_{m=1}^{\infty} \frac{J_1\left(\frac{a_m a}{R}\right) J_0\left(\frac{a_m r}{R}\right)}{J_0^2(a_m) a_m} \exp\left(-\frac{D t a_m^2}{R}\right) + n_0 \frac{(R^2 - a^2)}{R^2}; \quad (\text{"external" case}).$$

$J_1(\alpha_m) = 0$; α_m -Bessel function roots.

Computer simulations of these solutions gave the results plotted on fig. 7.

Fig. 7

i.e. the area where the reaction developed expands with time in the cross-section of the stream at the different lengths from the mixing point (nozzle tip). This effect, of course, must be taken into account for the secondary emission registration.

Since the shape of the stream is cylindrical when it intersects the plane waves from the laser, the optical density values will be different from those expected if the stream shape was rectangular. For a cylinder with the radius R_0 , absorption coefficient $\alpha(r)$, and refraction index n , the apparent optical density can be written in the form:

$$D = -\ln \int_0^{R_0} \exp \left\{ - \int_0^{\sqrt{R_0^2 - \frac{r^2}{n^2}}} \sqrt{z^2 + R_0^2 - 2Z \left(\sqrt{R_0^2 - \frac{r^2}{n^2}} \right)} dz \right\} \frac{dr}{R_0};$$

When the system is used as a cytometer, the absorbent area is situated at the central axis of the stream and has a radius r_0 . Transparent external sheath area has the radius R_0 , i.e.:

$$\alpha(r) = \begin{cases} \alpha, & r \leq R_0; \\ 0, & r > R_0; \end{cases}$$

For $n=1,33$; $r_0=75\mu\text{m}$; $R_0=400\mu\text{m}$; $\kappa=10\text{cm}^{-1}$

$$D=0,0275;$$

If the stream had a rectangular shape, an optical density, $D=0,0233$, would be expected for the same conditions. Thus, due to the effects of refraction, the "apparent" optical density for the cylindrical geometry would be higher (fig. 8).

Fig. 8

When the system is used as a microreactor, the function $\kappa(r)$ corresponds to the concentration distributions of the reagents. In the case of "total mixing" ($\kappa(r)=\text{const}$) :

$$D = -\ln \int_0^{R_0} \exp \left\{ -2\kappa \sqrt{\left(R_0^2 - \frac{r^2}{n^2} \right)} \right\} d\left(\frac{r}{R_0} \right) = 0,714;$$

For a rectangular stream using the same conditions, a $D=0.708$ would be expected. So in this case, the presence of cylindrical geometry increased the "apparent" optical density. Fresnel's deflection from the stream-air boundary leads to a negligible decrease of the same value (6,6%). It is clear that optical measurements from biological substances (e.g. fluorescence) often results in weak optical signals. That's why for our facility, a special light gathering system was designed. This system consists of spherical and elliptical mirrors allow increase the light gathering power of the system to about 50 % (fig. 9).

Fig. 9

4. CONCLUSION

In summary, we have described a new system that can function both as a flow cytometer and as a microreactor for studies of biochemical reaction kinetics. This system should be useful for cellular studies of genotoxicity and biodosimetry in exposed populations. Use of the system for studies of the primary elementary stages of biochemical processes and reactions, may also provide insights into the primary responses of biological systems from exposure to different environmental physical and chemical agents.

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6. REFERENCES

1. W.L.Bigbee, M.Vanderlaan, S.S.N.Fong, R.H.Jensen, "Monoclonal antibodies specific for the M and N forms of human glycophorin A", Mol. Immunology, 20, 1353-1362, 1985.
2. R.G.Langlois, W.L.Bigbee, R.H.Jensen, "Flow cytometric characterization of normal and variant cells with monoclonal antibodies specific for glycophorin A", J. Immunol. 134, 4009-4017, 1985.
3. R.G.Langlois, B.A.Nisbet, W.L.Bigbee, D.N.Rodinger, R.H.Jensen, "An improved flow cytometric assay for somatic mutations of the glycophorin A locus in humans", Cytometry 11, 513-521, 1990.
4. Seishi Kuozumi, Nori Nakamura, Masayuki Hakoda, Akio Awa, Michael Bean, Ronald H.Jensen, "Detection of somatic mutations at the glycophorin A locus in erythrocytes of atomic bomb survivors using a single beam flow sorter", Cancer Research 49, 581-588, 1989.
5. T.Straume, R.G.Langlois, J.Lucas, R.H.Jensen, W.L.Bigbee, A.T.Ramahlo, C.E.Brandao-Mello, "Novel biodosimetry methods applied to victims of Goiania accident", Health Physics, 60, 71-76, 1991.
6. R.H.Jensen, W.L.Begbee, R.G.Langlois, S.G.Grant, P.G.Pleshanov, M.A.Pilinskaya, A.Yu. Chirkov, "Laser-based flow cytometric analysis of genotoxicity of humans exposed to ionizing radiation during the Chernobyl accident", SPIE, Vol.1403, Laser Applications in Life Sciences, 1990, 372-380.
7. A.N.Martonosi, (ed.) "The enzymes of biological membranes", 1, Plenum Press, N.Y., London, 1985.
8. V.M.Agranovich, M.D.Galanin, "Perenos energii electronnogo vozbugdeniya v condensirovannich sredakh" (Russian), MFTI ed., Moscow, 1973.
9. Hammis, H., "Methods of Rapid Chemical Kinetics", MIR Publishers, Moscow, 1975.
10. L.A.Blumenfeld, P.G.Pleshanov, in:"Structure,dynamics and functions of biopolymers", Springer-Verlag, 1987, H.Fraunfelder, ed.
11. J.Gray, (ed.) "Flow Cytogenetics", Acad.Press, N.Y., London, 1989.

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