CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Standardization of Biochemical Profile of Mesenchymal Cell Materials by Probing the Level of Dehydrogenase Activity

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It is demonstrated that the output optical signal of MTT test is directly proportional to the number of viable cells in the primary culture of mesenchymal cells (skin fibroblasts and mesenchymal stem cells from the bone marrow, placenta, and umbilical cord). The slope of the best curve in coordinates "cell number – optical signal" reflecting specific productivity of MTT-formazan characterizes mean dehydrogenase activity of cells and their physiological activity. It was found that *in vitro* dehydrogenase activity of primary cultures of mesenchymal cells increased during the first 3-5 passages and then tended to decrease. The variant of MTT method presented here can be used for standardization of cell materials.

Key Words: *MTT method; primary cell cultures, mesenchymal stem cells; standardization*

Standardization of primary cell cultures and materials prepared from these cells is a necessary condition for their use in fundamental, preclinical, and clinical studies. Monitoring of biochemical profile, *i.e*. activity of key enzymes determining the rate of crucial intracellular processes, provides objective evaluation of viability and metabolic activity of cell cultures. Here we adapted the well-known MTT method of evaluation of dehydrogenase activity for standardization of the primary cultures of mesenchymal cells.

MTT method is based on the ability of live cells to absorb yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from the solution and reduce it to MTT formazan (MTT-F) forming purple-blue crystals (Fig. 1). The reaction of conversion of MTT into MTT-F is catalyzed by mitochondrial dehydrogenases and to a lesser extent, cytoplasm and non-mitochondrial membranes in the presence of NADH [11]. MTT test initially developed for evaluation of cytotoxicity and cell viability in suspension cultures [9] was shown to be applicable for adhesion cultures [1,2,4-6,8]. MTT and other tetrazolim salts are now widely used for evaluation of metabolic activity of cell of all taxonomic groups from microbes to mammals. Moreover, MTT test is used for evaluation of toxicity and tolerogenicity of antitumor drugs towards tumor and normal cells [12,14], cytotoxicity of nanoparticles [10], proapoptotic activity of various agents [13], viral infection of cells [5,6], and for other purposes.

Adaptation of MTT method for standardization of primary cultures of mesenchymal cells included the development of a method for probing dehydrogenase activity of these in culture, analysis of the dependence

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of optical density of extracted MTT-F (output optical signal) on the number of viable cells, and evaluation of changes in this parameter during consecutive passages of primary cultures of mesenchymal cells.

MATERIALS AND METHODS

Cultural plastics was purchased from Greiner.

Isolation of human skin fibroblasts. Skin fragments from adult donors were minced and incubated for 2 h at 37°C in 0.1% solution of type I collagenase (Gibco). The suspension was centrifuged, the pellet was resuspended in growth medium, transferred into Petri dishes, and cultured until 80% confluence (Fig. 2, *a*). Further culturing was performed in culture flasks.

Isolation of mesenchymal cells from human bone marrow. The cells obtained by separation of bone marrow aspirate from adult donors in Ficoll-urografin density gradient were transferred to culture flasks (10^4 cell/ml in growth medium) and growth until 80% confluence (Fig. 2, *b*). The growth medium was replaced every 3 days.

Isolation of mesenchymal cells from human umbilical cord. Human umbilical cord after normal delivery (gestation age 39-40 weeks) was washed with Hanks solution (PanEko) and after gentle mechanical stimulation incubated with 0.1% solution of type I collagenase (Gibco) for 30 min at 37°C. The suspension was centrifuged, the pellet was resuspended in growth medium, transferred into Petri dishes, cultured until 80% confluence (Fig. 2, *c*), and transferred into culture flasks for further culturing.

Isolation of mesenchymal cells from human placenta. Human placental amniotic tissues after normal delivery (gestation age 39-40 weeks) were washed with Hanks solution (PanEko) and after gentle mechanical stimulation incubated with 0.1% solution of type I collagenase (Gibco) for 30 min at 37°C. The suspension was centrifuged, the pellet was resuspended in growth medium, transferred into Petri dishes, and cultured until 80% confluence (Fig. 2, *d*). Further culturing was performed in culture flasks.

***In vitro* passaging of cells:** The cells were passaged routinely in culture flasks (Greiner) in a CO_2-incubator (37°C, 5% CO_2, 80% humidity). The growth medium consisted of DMEM-F12 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 ng/ml β-FGF (Gibco). The medium was changed 2 times a week. After attaining 80% confluence, the cells were suspended with trypsin-Versene (1:1, PanEko) and subcultured at 1:3 ratio.

The number of viable cells cell was determined routinely by exclusion of trypan blue in a Goryaev chamber.

MTT-test [5]. The cells were transferred to 96-well plates (8 wells contained 5000 cells, 8 wells contained 2500 cells, and 8 wells contained 1250 cells). After 6 h (after complete adhesion of cells to plastic), the medium was removed from wells and 100 µl serum-free culture medium and 10 µl MTT solution (10 mg/ml in PBS) were added to each well. The cells were incubated 1 h at 37°C and 7.5% CO_2. After incubation, the medium was carefully removed from the wells (without cells) and 100 µl DMSO was added for extraction of intracellular MTT-F (Fig. 2). Optical density was measured spectrophotometrically at λ=540 nm against 100 µl DMSO.

Construction of the best curve. The best curve for the experimental points was plotted using the method of least squares (Fig. 3): 24 pairs of experimental values (3 cell number values with 8 repeats for each) in the form of (N_i, D_i), where N is cell concntration, D is optical density in MTT method, and i=1, 2, …, 24, yielded a strain line:

$$D=a\times N+b;$$

$$a=\frac{24\times\left(\sum_{i=1}^{24}N_iD_i\right)-\left(\sum_{i=1}^{24}N_i\right)\times\left(\sum_{i=1}^{24}D_i\right)}{24\times\left(\sum_{i=1}^{24}N_i^2\right)-\left(\sum_{i=1}^{24}N_i\right)^2};$$

$$b=\frac{\left(\sum_{i=1}^{24}N_i^2\right)\times\left(\sum_{i=1}^{24}D_i\right)-\left(\sum_{i=1}^{24}N_i\right)\times\left(\sum_{i=1}^{24}N_iD_i\right)}{24\times\left(\sum_{i=1}^{24}N_i^2\right)-\left(\sum_{i=1}^{24}N_i\right)^2}.$$

The calculations were performed using Statistica 7 software (StatSoft Inc.).

RESULTS

Dehydrogenases converting MTT into MTT-F (Fig. 1) are a group of enzymes belonging to the family of oxidoreductases catalyzing electron transfer to the acceptor. Dehydrogenases participate in re-

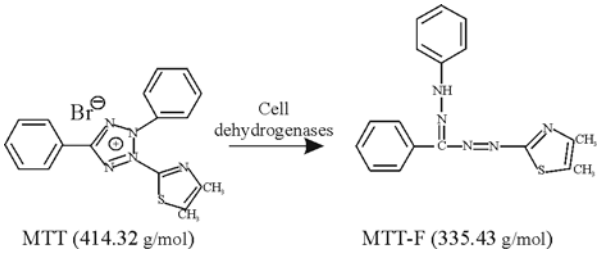

Fig. 1. Conversion of MTT into MTT-F under the effect of cell dehydrogenases.

actions of carbohydrate (glycolysis, pentose-phosphate pathway) and lipid (oxidation of fatty acids) metabolism and in cell respiration (tricarboxylic acid cycle and electron transfer to the cytochrome system). These enzymes are primarily localized in the cytoplasm and mitochondria [3,7,11]. MTT-F crystals are formed in the cytoplasm, but not in the nucleus (Fig. 2).

The optical density *D* measured in the MTT test reflects total dehydrogenase activity of the studied cell culture. Experiments for evaluation of the dependence of *D* on the number of N-cells in a well of 96-well plate repeated several times showed a significant direct proportion for all types of the test cell cultures: skin fibroblasts (FBT, Fig. 3, *a*), bone marrow MSC (Fig. 3, *b*), MSC from the umbilical cord (Fig. 3, *c*), and MSC from the placenta (Fig. 3, *d*). The slope of the *D*~*N* curve is proportional to dehydrogenase activity of the culture (per single cell), reflect optical density of MTT-F produced by one "average" cell, and can be specified as MTT-F-productivity. If culturing conditions remain constant, the production of MTT-F depends only on the functional state of the culture. Measuring the slope of *D*~*N* curve (Fig. 3) we can compare dehydrogenase activity of the cell culture at different passages (which cannot be done by comparing *D* only, because MTT test is performed under different conditions every time).

The dependence of MTT-F-productivity of cell passage showed changes in dehydrogenase activity and metabolic potential of the primary cell culture during *in vitro* processing of cell materials. After isolation of mesenchymal cells into culture *in vitro*, dehydrogenase activity (the slope of *D*~*N* curve) increases during the first few passages, but after passage 5 this parameter starts to decrease gradually (Fig. 4). This effect can be explained by the fact that immediately after cell isolation the culture is enriched with mature fibroblasts with reduced metabolism level. The percent of young metabolically active cells increases from passage to passage due to elimination of mature cells from the culture. However, later the percent of metabolically active cells decreased again, which led to a decrease of dehydroge-

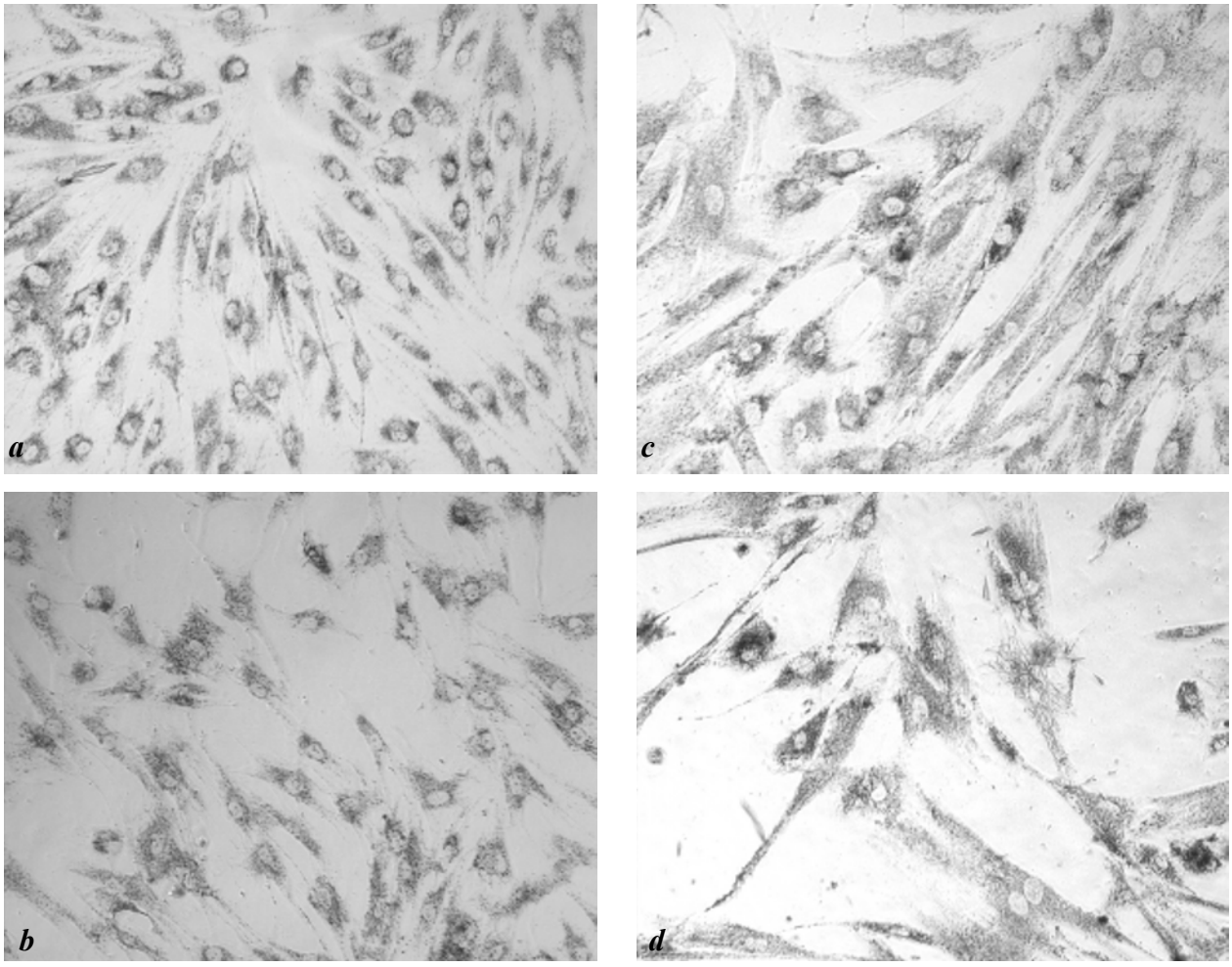

Fig. 2. MTT-F crystals in the cytoplasm of skin FBT (*a*), MSC from the bone marrow (*b*), MSC from the umbilical cord (*c*), MSC from human placenta (*d*) of passage 4 of *in vitro* culturing.

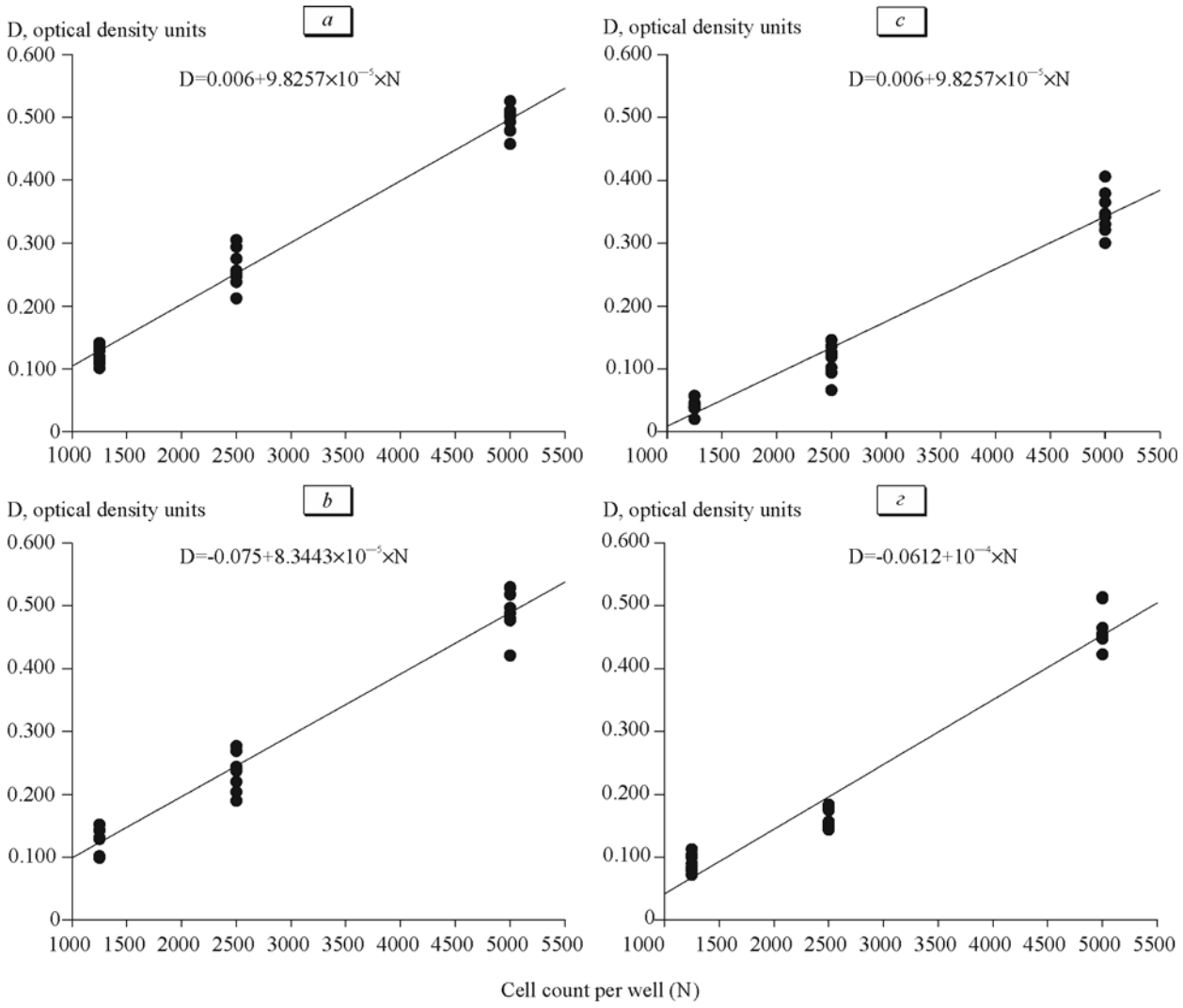

Fig. 3. Best curves plotted using the method of least squares for MTT test data set (8 repetitions for each point) for different concentrations of human skin FBT (*a*), MSC from human bone marrow (*b*), MSC from human umbilical cord (*c*), MSC from human placenta (*d*) of passage 4 of *in vitro* culturing.

nase activity and MTT-F-productivity in the MTT test.

Cultures of passages 3-6 (peak of dehydrogenase activity) are most suitable for preparation of cell materials. These data agree with empirical rule: cultures elder than passage 5 should not be used for preparation of cell materials; otherwise medical application of such cultures can be low effective.

Thus, MTT test is a convenient and effective method of probing of metabolic activity of primary cultures of mesenchymal cells. An advantage of this method is the possibility of direct using MTT test for adherent cultures (without resuspension of the cells). This is an important advantage, which is seen from attempts to develop new methods of culture evaluation not requiring cell removal from the substrate.

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REFERENCES

1. V. V. Burunova, N. E. Manturova, G. O. Smirnova, *et al., Russ. Med. Zh.*, **17**, No. 17, 1058-1062 (2009).
2. V. V. Burunova, Yu. G. Suzdal'tseva, A. V. Voronova, *et al., Kletochn. Tekhnol. Biol. Med.*, No. 2, 97-101 (2008).
3. R. Murray, D. Granner, P. Mayes, and B. Roduell, *Human Biochemistry* [Russian translation], Moscow (2004).
4. Yu. V. Suzdal'tseva, V. V. Burunova, I. V. Vakhrushev, *et al., Kletochn. Tekhnol. Biol. Med.*, No. 1, 3-10 (2007).
5. M. Yu. Schelkanov, V. F. Eremin, I. B. Sakhuriya, *et al., Biokhimiya*, **64**, No. 4, 431-436 (1999).
6. M. Yu. Shchelkanov, I. B. Sakhuriya, V. V. Burunova, *et al., Immunologiya*, No. 1, 37-41 (1999).
7. E. Pearse, *Histochemistry Theoretical and Applied* [Russian translation], Moscow (1962).

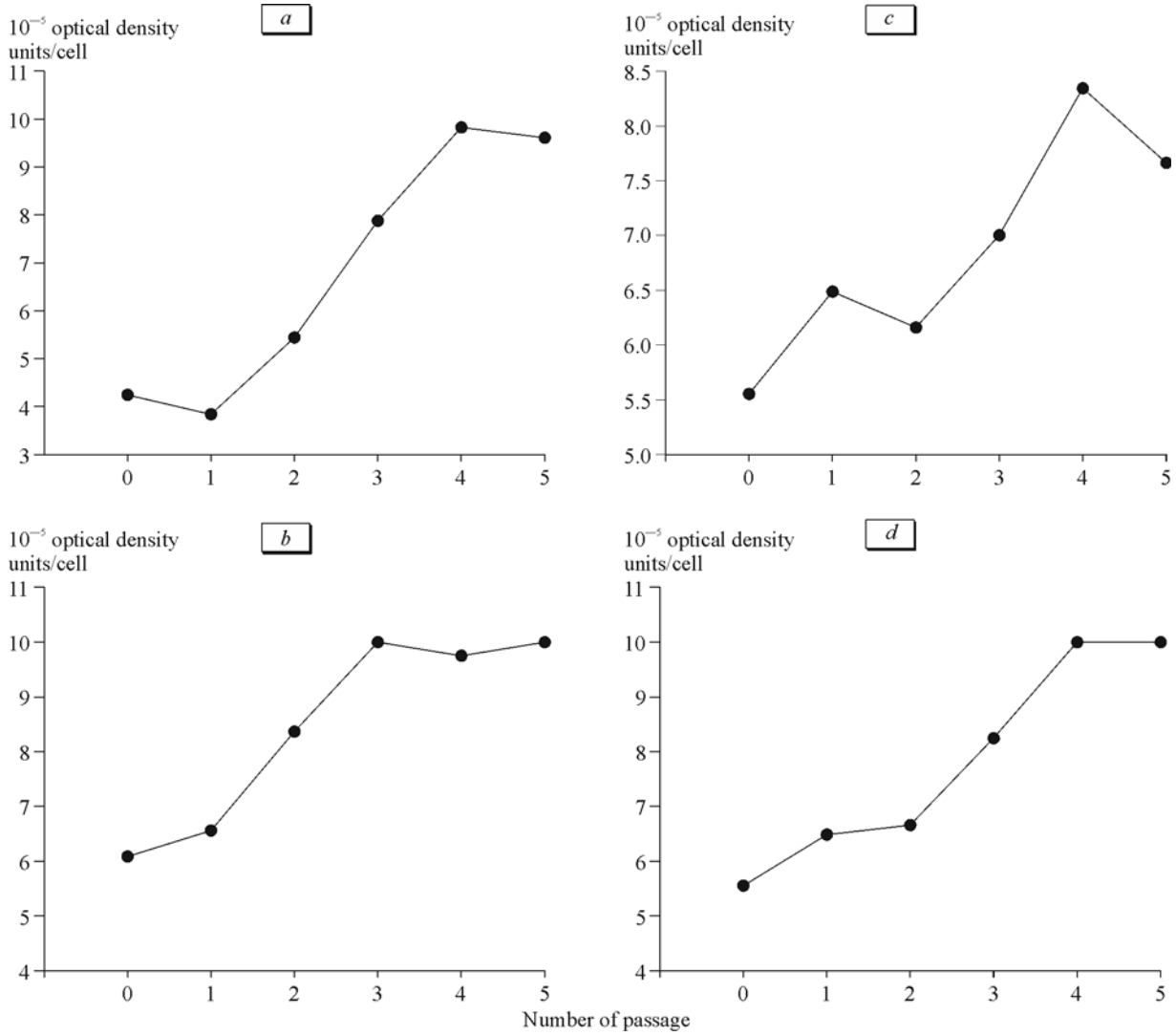

Fig. 4. MTT-F productivity as a function of passage number for different primary cultures of mesenchymal cells. *a*) skin FBT, *b*) MSC from bone marrow, *c*) MSC from umbilical cord, *d*) MSC from placenta. Ordinate: MTT-F productivity.

8. K. N. Yarygin, Yu. G. Suzdal'tseva, V. V. Burunova, *et al, Kletochn. Tekhnol. Biol. Med.*, No. 1, 53-59 (2006).
9. T. Mosmann, *J. Immunol. Methods*, **65**, Nos. 1-2, 55-63 (1983).
10. S. Ahmadian, J. Barar, A. A. Saei, *et al., J. Vis. Exp.,* **26**, 1191 (2009).
11. M. V. Berridge, P. M. Herst, A. S. Tan, *Biotechnol. Annu Rev.*, **11**, 127-152 (2005).
12. T. Hayon, A. Dvilansky, O. Shpilberg, and I. Nathan, *Leuk. Lymphoma*, **44**, No. 11, 1957-1962 (2003).
13. D. T. Loo and J. R. Rillema, *Methods Cell. Biol.*, **57**, 251-264 (1998).
14. J. M. Sargent, *Recent Results Cancer Res.*, **161**, 13-25 (2003).