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Letter to the Editor

SIZE CHANGES IN DIFFERENTIATING NEUROBLASTOMA CELLS

Dear Editor:

The nervous system presents a challenge to the field of tissue engineering because much of its complex neurochemical and neuroanatomical architecture has only recently been understood (Bellamkonda and Aebischer, 1994). Improvements in our understanding of the molecular control of cell replication and differentiation is paving the way for a number of possible neural tissue engineering applications. Past work has evaluated a differentiating murine neuroblastoma cell line (N1E-115) as a neural cell-based system for *in vitro* toxicology and efficacy testing (*see* Hernandez and Kisaalita, 1996; Kisaalita and Bowen, 1996, 1997). In these studies, differentiation was characterized by resting membrane potential (V_m) establishment; the relative changes in V_m were measured by flow cytometry with the aid of a voltage-sensitive oxonol dye. Forward-angle light scatter (FALS), one of the flow cytometry parameters, is defined as light of the same wavelength as the illuminating laser beam that is refracted as it passes through the cell so as to diverge from the original path of the laser beam by approximately 0.5°. Hence, FALS is dependent on both cell refractive index and size. The relative distribution of differentiating/differentiated cells between low- and high-FALS has been proposed as a potential culture electrophysiological differentiation index before and after terminal differentiation (Kisaalita and Bowen, 1997). Our purpose here is to study the relationship between cell-size distribution and culture age for differentiating and control cells, and perhaps more importantly, assess the effect of cell differentiation size changes to FALS. How cell size changes influence FALS has biological relevance when using FALS as a measure of the extent of electrophysiological differentiation (Kisaalita and Bowen, 1997).

TABLE 1

MANN-WHITNEY *U* TEST COMPARISON OF CONTROL (NONINDUCED) AND TREATMENT N1E-115 MURINE NEUROBLASTOMA CELL SIZE AREAS[a]

	Day 1 treat.	Day 2 treat.	Day 3 treat.	Day 5 treat.	Day 6 treat.	Day 8 treat.	Day 10 treat.	Day 12 treat.
Day 1 control	0.0001[b] reject H_0	0.4361 don't rej. H_0	0.0001 reject H_0	0.1749 don't rej. H_0	0.0001 reject H_0	0.0001 reject H_0	0.0357 reject H_0	0.0783 don't rej. H_0
Day 2 control	0.0001 reject H_0	0.0127 reject H_0	0.0002 reject H_0	0.6755 don't rej. H_0	0.0004 reject H_0	0.0013 reject H_0	0.2137 don't rej. H_0	0.2660 don't rej. H_0
Day 3 control	0.0001 reject H_0	0.8044 don't rej. H_0	0.0001 reject H_0	0.0003 reject H_0	0.9658 don't rej. H_0	0.4891 don't rej. H_0	0.0388 reject H_0	0.0402 reject H_0
Day 5 control	0.0001 reject H_0	0.0067 reject H_0	0.0139 reject H_0	0.4593 don't rej H_0	0.0006 reject H_0	0.0021 reject H_0	0.3034 don't rej. H_0	0.4353 don't rej. H_0
Day 6 control	0.0001 reject H_0	0.0261 reject H_0	0.0502 don't rej. H_0	0.4437 don't rej. H_0	0.0001 reject H_0	0.5499 don't rej. H_0	0.0492 rejct H_0	0.0395 reject H_0
Day 8 control	0.0024 reject H_0	0.0008 reject H_0	0.0021 reject H_0	0.3832 don't rej. H_0	0.0968 don't rej. H_0	0.0040 reject H_0	0.1508 don't rej. H_0	0.1241 don't rej. H_0
Day 10 control	0.0001 reject H_0	0.0694 don't rej. H_0	0.1064 don't rej. H_0	0.8403 don't rej. H_0	0.7617 don't rej. H_0	0.3798 don't rej. H_0	0.2170 dpn't rej. H_0	0.9328 don't rej. H_0
Day 12 control	0.0001 reject H_0	0.5572 don't rej. H_0	0.4714 don't rej. H_0	0.0001 reject H_0	0.0005 reject H_0	0.0001 reject H_0	0.0081 reject H_0	0.0001 reject H_0

[a]Blocks below and above the main diagonal compare control vs. control and treatment vs. treatment, respectively, across the day of study.

[b]*p*-values for *Z*-test.

N1E-115 cells of Passage 12 were obtained from Dr. M. Nirenberg, National Institutes of Health (Bethesda, MD). Previously published protocols for N1E-115 cell cultures (Kimhi et al., 1976; Moolenaar and Spector, 1978; Miyake and Kurihara, 1983) were followed. Briefly, N1E-115 cells were routinely cultured in 37° C air plus 10% CO_2 at 90% relative humidity. The growth media was composed of Dulbecco's modified Eagle's medium (DMEM) containing 0.37% $NaHCO_3$ (wt/vol) and supplemented with 13% fetal bovine serum (FBS), 50 units/ml penicillin, 50 g/ml streptomycin, and 2 m*M* glutamine. Cultures were monodispersed gently by flushing the confluent cells from the base of a 75-cm² T-flask (Costar, Cambridge, MA) by a stream of medium ejected from a Pasteur pipette. The suspensions were centrifuged (500 g, 10 min) and the pellet resuspended in 25-cm² T-flasks (Costar) with fresh growth media at 2.0×10^6 viable cells (able to exclude trypan blue) per flask, unless otherwise stated. Flasks (in triplicate) were incubated for 24 h to allow cells to settle and adhere to the base of the flask. Cells were then exposed to the differentiating medium (this is the same as the growth medium except serum was reduced to 0.5%). The differentiating medium was changed every 3 to 4 d. Cell-size data were taken at Days 1, 2, 3, 5, 6, 8, 10, and 12 after exposure to the differentiating medium. Kisaalita and Bowen (1997) have previously shown that it takes 12 d for N1E-115 cells to mature and exhibit high steady V_m. A control culture that was not induced to differentiate was included for each day of the analysis.

Cells were monodispersed, centrifuged (500 g, 10 min), resuspended in a saline solution, and counted with a hemocytometer. The saline solution contained (in m*M*): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 N-[2-Hydroxyethyl]piperazine-N'[2-etha-nesultonic acid] (HEPES), 10 D-glucose, pH 7.5. Cells were washed (500 g, 10 min) and resuspended at 0.5×10^6 cells/ml. Flow cytometry analysis of the cell suspension was performed with the University of Georgia (UGA) Research Services' Coulter EPICS 753 (Hialeah, FL) dual beam instrument. FALS and side-light scatter (SSC) signals were obtained in response to argon ion laser excitation (488 nm) at 200

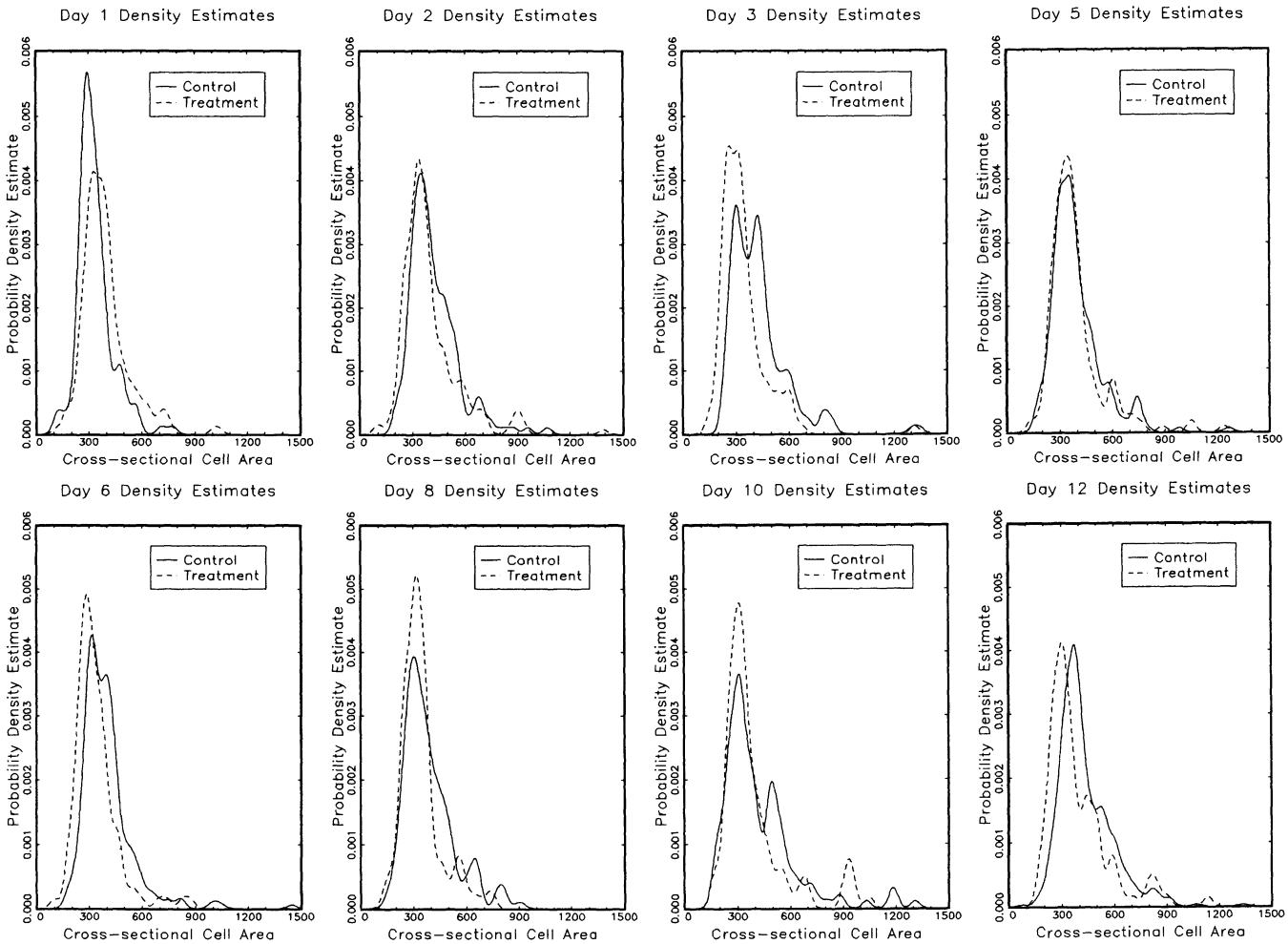

FIG. 1. Comparison of cell size kernel density estimates for control and treatment N1E-115 cells. Cross-sectional area is in μm^2.

mW power output (Coherent, Palo Alto, CA). SSC is defined as light of the same wavelength as the illuminating laser beam that bounces off in the direction orthogonal to the line of the laser. SSC signals were recorded by PMT_4 and FALS signals were linearly amplified with a gain of 2. Fifty thousand events were counted at an approximate rate of 200 events per second. Subcellular debris were gated out on the basis of their low FALS. In order to compare results for experiments conducted at different times, polystyrene fluospheres (Coulter Corp., Hialeah, FL) were used to set PMT_4 (SSC signal) at channel number 100 ± 2.

Aliquots (0.1 ml) of monodispersed cells were diluted 1:1 with 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO). A few drops (approximately 0.05 ml) of each mixture were transferred to a hemocytometer. Nonviable cells were stained blue-purple and viable cells did not take up the dye. A trypan blue stained cell suspension was transferred to a hemocytometer and viewed under an inverted light microscope (IMT2, Nikon) at $\times 200$ magnification. Using Olympus QUE-3 color image system, sufficient images were acquired to include at least 97 nonstained (viable) cells. A conversion utility (Scott Data Inc., Tonka Bay, MN) was used to convert QUE to TIFF files. Cell cross-sectional areas were extracted from TIFF images with a user-defined macro in OPTIMAS image analysis software (Optimas, Edmond, WA).

We now consider the question of whether or not the control and differentiated (henceforth termed treatment) cell-size distributions, as characterized by cross-sectional area, are identical over the 12-d study period. Our interest lies in comparing the entire cell size distributions; hence, standard two-sample z and t tests, generally used to compare population means only, are inadequate for our purposes.

The nonparametric Mann-Whitney U test, described in detail in Hogg and Craig (1995), will first be used to test whether or not the control and treatment cultures were drawn from statistically identical populations. Table 1 summarizes 64 different Mann-Whitney U tests. Each block in Table 1 considers a test of the null hypothesis H_0: the two cell size area populations are identically distributed versus the alternative hypothesis H_1: the two cell size area populations are not identically distributed; p-values for each test, computed from the standard normal distribution, are reported.

The blocks below the main diagonal in Table 1 compare control cultures only; for example, the very small p-value of 0.0001 in the first column of the second row strongly suggests that the control culture cell sizes differ statistically from Day 1 to 2. Analogously, the

blocks above the main diagonal in Table 1 compare treatment cultures on different days only. The main diagonal, and perhaps the most important component of Table 1, considers tests of treatment versus control on each of the 8 d considered in the study.

Thirty six of the 64 tests in Table 1 result in rejection of H_0 at the 5% level; this conclusion is indicated in each block. These 36 rejections greatly exceed the $64(.05) = 3.2$ expected under the assumption that the control and treatment cell size areas are distributionally equivalent and that neither control or treatment cell size areas change distributionally with the day of study. Hence, our overall conclusion is that some of the treatment and control cell size areas differ statistically.

Inspection of the main diagonal of Table 1 shows that six of the eight tests reject H_0 at the 5% level; these six rejections greatly exceed the $8(.05) = .4$ expected. The probability of getting six or more rejections is 4×10^{-7} assuming that H_0 is indeed true in all tests. Because the main diagonal of Table 1 compares treatments with controls on the same study day, we conclude that the treatment and control cell size areas indeed differ on some days in the study.

While the above Mann-Whitney U tests indicate that treatment and control cultures differ, they do not suggest how they differ. To investigate this aspect further, a kernel density function estimation technique as described in Silverman (1986) was used to graphically analyze the cell size areas. Kernel density estimates can be interpreted as histograms that are smoothed to eliminate unnatural fluctuations.

Fig. 1 compares control and treatment kernel density estimates of cell sizes, computed with a standard normal kernel function and a "bandwidth" parameter of 25. The density estimates for the treatment cultures, on all but Days 1, 5, and 10, are shifted to the left of the control density estimates. This suggests that the treatment cells are smaller than the control cells in a distributional sense during Days 2, 3, 6, 8, and 12. On Days 5 and 10, the control and treatment density estimates appear similar. Day 1 is a noted exception to the above pattern as the treatment density estimate is shifted to the right of the control density estimate. Finally, note that the treatment cell sizes on Day 12, the last day of the study, appear to have undergone the largest size reduction (the possible exception of Day 3

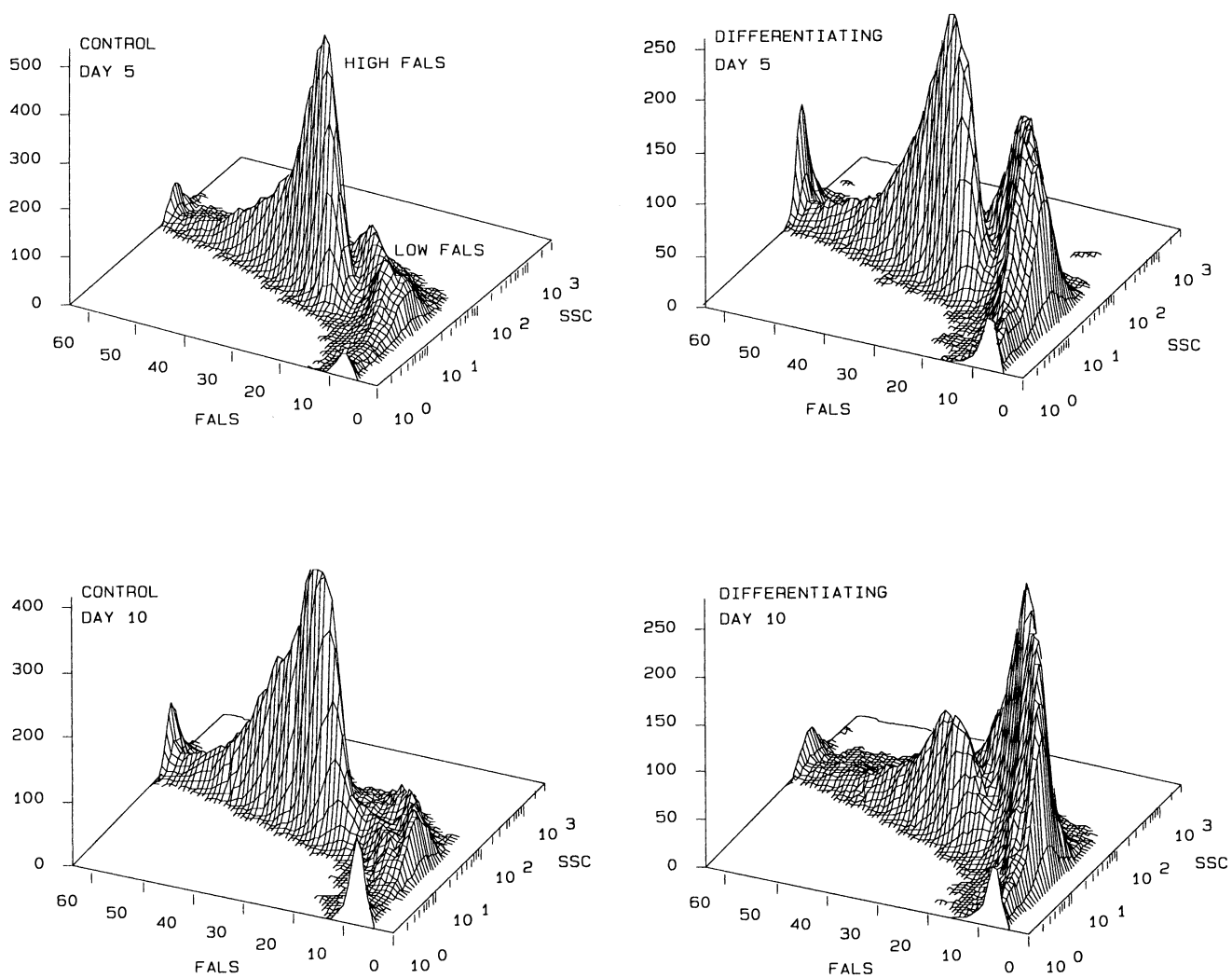

FIG. 2. Relative distribution of cells in high- and low-FALS region for control and differentiating N1E-115 cells. Vertical scale represents number of events (cells).

is noted). The overall conclusion is to support the assertion that the treatment cells have undergone some size reduction.

It is worth noting that a typical Gaussian (normal) analysis of the data would be inappropriate. The density estimates in Fig. 1 show that the data is frequently skewed with a variety of modal features. A small percentage of large cells (less than 5%) were found in both the control and treatment cultures. The presence of these cells gives rise to the "right-most" modes in the cell-size density estimates ($>$ 600 μm^2). The most predominant large cells were those with differentiated morphology (i.e., with neurite extensions).

Overall, the results show that N1E-115 cell size distributions change with both time and culture type (control and treatment). It should be pointed out that, since the amount of probe absorbed by cells is dependent on cell sizes, knowledge of cell-size distribution profiles is useful for quantitative determination of V_m by voltage-sensitive probes.

Several prior investigators have associated N1E-115 cell-size increases with differentiation (Peacock et al., 1972; Kimhi et al., 1976; Santone et al., 1986). This discrepancy is attributed to differences in techniques for cell size determination. In this study, cells were monodispersed and observed under conditions close to free suspension. In the previous studies, cells were observed while attached to substrates. It is well known that growth and differentiation of cells frequently follows a two-step process (Ramsden et al., 1993). First, cells interact with surfaces by attaching while maintaining the round shape they possess in suspension. Second, attachment is followed by a type of conformation change (spreading) in which the cells increase their area in contact with the surface. It is likely that previously reported cell-size increases were due to spreading. This explanation is consistent with the earlier observation by Moolenaar et al. (1981) that one of the responses of N1E-115 cells to serum removal was flattening of the cell body. Compelling evidence suggesting that cell-size change had negligible effect on FALS was provided by the Day 5 and 10 results where H_0 was accepted: as shown in Fig. 2, the distributions of cells into low- and high-FALS regions as previously defined (Kisaalita and Bowen, 1997) were different for control and differentiating cells, yet the corresponding cell-sizes were statistically accepted as identically distributed (Table 1). Therefore, decreases in N1E-115 culture FALS with age is most likely due to electrophysiological differentiation only.

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