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# Human neuroblastoma (SH-SY5Y) cell culture and differentiation in 3-D collagen hydrogels for cell-based biosensing

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**Abstract**

Cell-based three-dimensional systems are desirable in the field of high throughput screening assays due to their potential similarity to in vivo environment. We have used SH-SY5Y human neuroblastoma cells cultured in 3-D collagen hydrogel, confocal microscopy and immunofluorescence staining, to assess the merit of the system as a functional, cell-based biosensor. Our results show differences between 2-D and 3-D resting membrane potential development profile upon differentiation. There was no statistically significant difference in SH-SY5Y proliferation rate between 2-D monolayer and 3-D collagen culture formats. A large percentage of cells (2-D, 91.30% and 3-D, 84.93%) did not develop resting membrane potential value equal to or lower than −40 mV; instead cells exhibited a heterogeneous resting membrane potential distribution. In response to high $K^+$ (50 mM) depolarization, 3-D cells were less responsive in terms of increase in intracellular $Ca^{2+}$, in comparison to 2-D cells, supporting the hypothesis that 2-D cell calcium dynamics may be exaggerated. L-Type $Ca^{2+}$ expression levels based on staining results was inconsistent with Bay K 8644 channel activation results, strongly suggesting that either the majority of the channels were non-functional or could not be activated by Bay K 8644. In general, the results in this study confirm the depolarization-induced differences in intracellular calcium release when cultured using a 2-D versus a 3-D matrix.


*Keywords:* Collagen hydrogel; SH-SY5Y; Voltage gated calcium channels; Resting membrane potential; Confocal microscopy

## 1. Introduction

Voltage gated ion channels are emerging as essential drug targets in the pharmaceutical industry due to their impact on diseases of the central nervous and cardiovascular systems (Denyer et al., 1998; Gonzalez et al., 1999). With the current advance in genomics and combinatorial chemistry, it is now possible to screen thousands of compounds against voltage gated ion channel targets in a 96- or 384-well plate in a short time (Denyer et al., 1998). However, in vitro biochemical assays target specific enzymes or proteins associated with the ion channel and as a result the "hits" generated from such screens often fail when tested in the natural and complex environment of an organism (O'Connor et al., 2000a; Durick and Negulescu, 2001). Thus, in order to produce physiologically meaningful results, it is essential to study the ion channels in living cells (Gonzalez et al., 1999; O'Connor et al., 2000a; Durick and Negulescu, 2001).

Cell-based biosensors prove advantageous because they provide a cell with all of the necessary biological interactions that are available to a cell in its in vivo environment. The biosensors can be utilized to study biological and chemical warfare agents, environmental toxins, or to detect possible deleterious effects of a drug before clinical trials (O'Connor et al., 2000a; Durick and Negulescu, 2001). Many cell-based biosensors in use today are based on a flat, two-dimensional glass or plastic surface that may not produce results characteristic of in vivo (Cukierman et al., 2002; O'Connor et al., 2000a). Extracellular matrix (ECM) is the three-dimensional substrata, which provides a direct interaction between cells via integrin receptors in vivo. These ECM mediated receptor

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cell interactions are responsible for the regulation of cell proliferation, migration, and adhesion (Friedl and Brocker, 2000). Recent studies have shown that performing cell-based assays in three-dimensional substrates may provide physiologically relevant results (Cukierman et al., 2001; Friedl and Brocker, 2000). Thus, many three-dimensional substrates are being considered to address the need to study cell behavior in an "in vivo" environment. Examples of 3-D substrates include microgravity bioreactors (Jessup et al., 1993), electrospun nanofibers (Matthews et al., 2002), micro- and nano-structured substrata (Powers et al., 2002), and natural and synthetic scaffolds (Sherwood et al., 2002; Deng et al., 2002).

Collagen hydrogel has been used in various three-dimensional cell behavior studies (Cukierman et al., 2001; O'Connor et al., 2000b). In a previous paper, Mao and Kisaalita (2004a) studied voltage gated calcium channel (VGCC) properties of the neuroblastoma cell line, IMR-32 in a 3-D collagen hydrogel, and concluded that the optical and mechanical properties of collagen hydrogel (0.5–1.0 mg/ml collagen) are suitable for a cell-based biosensor. Furthermore, Mao and Kisaalita (2004a,b) showed a significant difference in calcium response to high $K^+$ depolarization between cells grown in 2-D flat dishes (monolayer) versus 3-D hydrogel. Day 13 cells (differentiated) in 3-D collagen gel showed a calcium response, however, Day 2 cells (undifferentiated) did not. Unfortunately, IMR-32 cells do not develop a resting membrane potential ($V_m$) characteristic of nerve cells (Rao and Kisaalita, 2001). Also, Mao and Kisaalita (2004a) comparative proliferation results were not conclusive. The purpose of this paper is to extend the study to a human neuroblastoma cell line that develops a neuronal-like resting membrane potential, and to compare cellular proliferation between the traditional 2-D and 3-D collagen hydrogel.

## 2. Materials and methods

### 2.1. Cell line and cell culture

SH-SY5Y cell line was chosen because it has been shown to develop a more characteristic resting membrane potential (Sonnier et al., 2000) and to possess voltage gated calcium channels upon differentiation (Morton et al., 1992; Reuveny and Narahashi, 1993; Seward and Henderson, 1990; Reeve et al., 1995). Also, it has a short doubling time and it is capable of differentiating into different neuron-like subtypes. SH-SY5Y cell line was cultured in 75-$cm^2$ tissue culture flasks (Costar, Cambridge, MA) with 30 ml growth medium at 37 °C in a 10% $CO_2$ humidified atmosphere. Growth medium was made with minimum essential medium with 10% heat inactivated fetal bovine serum (FBS), 2.2 g/l sodium bicarbonate, and 2 mM L-glutamine (Mao and Kisaalita, 2004a). Growth medium was replaced every other day and cells were passed at 75% confluence.

#### 2.1.1. 2-D culture

For monolayer or 2-D culture, cells were plated on No. 1.5 glass coverslip embedded in a 35-mm petri dish (MatTek Co., Ashland, MA) at a density of $5 \times 10^5$ cells per plate. The cells were allowed to grow for 2 days, and then growth medium was replaced with differentiation medium made of 5% FBS, 2.2 g/l sodium bicarbonate, 2 mM L-glutamine, and 200 nM TPA or 1 mM dibutyryl cAMP and 2.5 μM 5-bromodoexyuridine (Mao and Kisaalita, 2004a). The differentiation medium was changed daily.

#### 2.1.2. 3-D collagen-cell culture

Twelve milligrams collagen (Rat tail tendon, Type I, Sigma, St. Louis, MO) was dissolved in 2.5 ml 0.2% (v/v) acetic acid and sterilized under UV overnight in a biosafety hood. The next day, 2.5 ml 2× PBS, 35 μl 1 M NaOH, and 7 ml 1× PBS was added to the solution to obtain a final collagen solution of 1 mg/ml at pH 7.4, which was stored at 4 °C to avoid gel formation (Mao and Kisaalita, 2004a). SH-SY5Y cells at a density of $1.2 \times 10^6$ cells/ml were mixed with 1 ml collagen solution and spread on a 35-mm petri dish with an embedded No. 1.5 glass coverslip. The plates were incubated at 37 °C for 2 h to allow gel formation, and then 2 ml growth media was added to the plates. The growth medium was replaced with differentiation medium after 2 days and replaced daily thereafter.

### 2.2. Proliferation

Cell proliferation was measured in both 2-D and 3-D over 72 h. For 2-D cultures, 500,000 cells were plated in 3 wells each of a 6-well plate and 3 ml growth media was added to each well. Cells were allowed to grow for 24 h after which they were dislodged mechanically, centrifuged, and re-suspended in growth media. Cells were counted using a hemocytometer. This procedure was followed after 48 and 72 h. For 3-D cultures, $1.2 \times 10^6$ cells were plated in 35 mm petri plates each according to the procedure described above for 3-D cultures. Cells were released using 3 mg of collagenase Type I (Sigma) dissolved in 1 ml of DMEM. Cells were counted as described above and data was fitted to the growth curve below (Eq. (1)):

$$Y = Ae^{kt} \tag{1}$$

where $k$ is the growth rate, $A$ the number of cells at the start of the experiment and $Y$ is the number of cells at time, $t$.

### 2.3. Resting membrane potential ($V_m$) development

We have previously used a voltage-sensitive dye, oxonol (Kisaalita and Bowen, 1997), to assess the resting membrane potential of cells; unfortunately, it was not possible to perform measurements at the single cell level. In this study, we have used a confocal microscopy method used to determine resting membrane potential, at the single cell level that was previously described by Mao and Kisaalita (2004b) in detail.

Briefly, the potentiometric fluorescent dye, tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Eugene, OR) was used. It distributes across the plasma membrane in a membrane potential dependent manner. The dye binds in a non-specific manner, which provides better visualization of cellular components. Days 2 and 8 monolayer cells were washed with 2 ml HEPES buffered saline (HBS) twice and incubated with 2 ml HBS containing 0.5 μM TMRM for 20 min at 37 °C in a humidified incubator maintained at 10% $CO_2$. On Days 2 and 8, cells embedded in collagen hydrogel were loaded with TMRM for 40 min. Cells were excited with 543 nm Green HeNe laser (PCM 2000, Nikon) and the emission was captured through a 565 nm long-pass filter by a photomultiplier detector. Simple PCI software (Compix Inc., Cranberry Township, PA) was used to capture and store images.

### 2.4. Calcium imaging

A membrane permeable dye, calcium green-1, acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR) was used to visualize calcium influx into single cells upon depolarization with high $K^+$ buffer. Monolayer cells on Days 2 and 8 were washed with HBS and loaded with 5 μM calcium green-1 in 1 ml HBS containing 3% heat inactivated FBS and 0.02% Pluronic F-127. The plates were incubated at 37 °C for 1 h. Cells were washed with HBS twice and allowed to de-esterify for 1 h in a 37 °C incubator. On Days 2 and 8, collagen-embedded cells were loaded with a high dye concentration, 120 μM and allowed to incubate for 5 h. Calcium green-1 was excited with 488 nm argon laser and the emission was captured through a 515 nm long pass filter. Cells were depolarized with 100 μl of high $K^+$ buffer at a final concentration of 50 mM $K^+$. Change in calcium influx was recorded continuously.

The dihydropyridine agonist, Bay K 8644 (5–25 μM), was used to induce the opening of L-Type of VGCC on Days 2 and 8 monolayer cells as well as Day 8 3-D cells. The calcium influx was recorded as described above and the number of cells with VGCC activation in both monolayer and 3-D cultures were compared.

### 2.5. Immunofluorescence

L-Type channels were stained with BODIPY-FL verapamil (B7431, Molecular Probes). N-Type channels were stained with anti-calcium channel ($\alpha_{1B}$ subunit) antibody (C1478, Sigma) and detected by Alexa Fluor 488-conjugated chicken anti-rabbit antibody. The procedure used for both monolayer and collagen entrapped cells has been outlined in detail elsewhere (Nakamura et al., 2003) and is briefly described below.

#### 2.5.1. N-Type channels

Briefly, Days 2, 8, and 13 cells for both monolayer and 3-D cultures were fixed with 4% formaldehyde for 30 min at room temperature. Cells were allowed to air dry and 1% Triton X-100 was added for 30 min. Cells were washed with PBS and incubated in PBS/1% BSA for 30 min. Anti-calcium channel antibody (10 μg/ml) was added to plates for N-Type staining for 1 h. Cells were washed with 0.1% Tween 20. Cells were incubated with Alexa Fluor 488 conjugated antibody (10 μg/ml) for 1 h and washed again with 0.1% Tween 20. Numerous images of stained cell preparations were captured with the Nikon PCM 2000 confocal imaging system. Samples were excited with the argon laser and gray levels were measure for all the cells in view using Simple PCI software (Compix Inc., Cranberry Township, PA).

#### 2.5.2. L-Type channels

The above described procedure was followed for staining L-Type channels with the exception that cells were loaded with BODIPY-FL verapamil (10 μg/ml) after the incubation with PBS/1% BSA. Cells were then washed with 0.1% Tween 20 and imaged as described above. In order to calculate the number of cells with channels, a procedure applied to flow cytometry data was adopted (Traill et al., 1986). The means of gray level for each set of data were calculated and cells with gray levels in the 75th percentile or higher were scored as having channels. Although arbitrary, the choice of 75th percentile for comparative purposes has proved very sensitive when only small differences exist among cells (Traill et al., 1986). The total number of cells possessing each type of channel was determined for Days 2, 8, and 13 and statistical comparisons were made for both 2-D and 3-D cultures.

## 3. Results and discussion

### 3.1. Proliferation

Cell proliferation rate was measured in both 2-D and 3-D cultures up to 72 h after plating. Fig. 1 shows plots of 2-D and 3-D cell numbers. The value of the growth rate, $k$, for 2-D was $0.0181 \pm .0034$ ($k \pm$ standard error), resulting in a doubling time of 38.3 h; in 3-D, the value of $k$ was $0.0193 \pm 0.0036$ ($k \pm$ standard error), resulting in a doubling time of 35.9 h. No statistically significant difference was found between the two doubling times ($p \leq 0.4175$). Although many studies support the conclusion that collagen increases the rate of proliferation (Kleinman et al., 1981; Ignatius et al., 2005), others have suggested that collagen decreases the rate of proliferation (Senoo et al., 1996). It has also been suggested that whether collagen increases cell proliferation depends on the particular cell type (Kleinman et al., 1981).

### 3.2. Resting membrane potential development

TMRM is a potentiometric dye that redistributes across the plasma membrane in a potential-based manner (Ehrenberg et al., 1988). The Nernst equation can be used to determine the resting membrane potential based on the difference

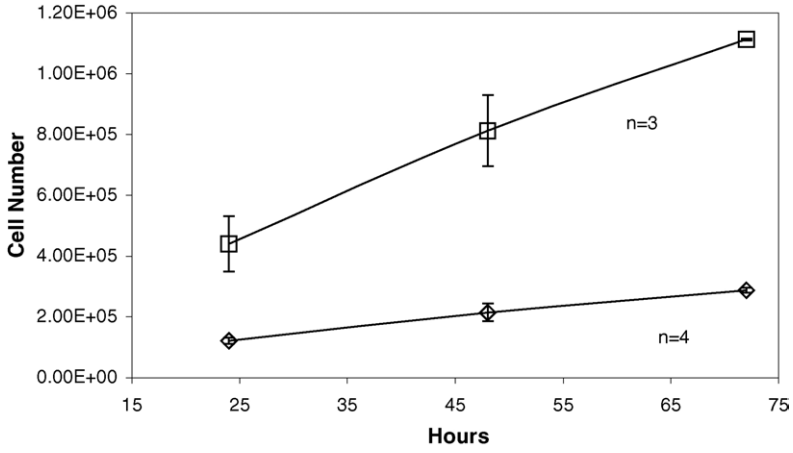


Fig. 1. Cell growth in 2-D (◊) and 3-D (□) cultures. Cells in collagen hydrogel were released with collagenase (3 mg/ml). Cells were counted with a hemocytometer. The error bars are one standard deviation.

between inside and outside fluorescent dye concentration (Loew, 1998). Mao and Kisaalita (2004a) used a modified form of the Nernst equation (Eq. (2)) that corrected for background values, error in measuring extremely low extracellular dye concentrations, and non-specific binding of the dye:

$$V_m = -58 \log 10 \left[ \frac{(F_{in}^{10\%} - B^{10\%}) \times (F_{out\_free}^{100\%} - B^{100\%})}{(F_{out}^{100\%} - B^{100\%}) \times (F_{in\_free}^{10\%} - B^{10\%})} \right] \quad (2)$$

where $F_{in}^{10\%}$ is the intracellular fluorescence intensity at 10% power, $B^{10\%}$ the background value at 10% power, $B^{100\%}$ the background value at 100% power, $F_{out\_free}^{100\%}$ the extracellular fluorescence correction factor at 100% power, $F_{out}^{100\%}$ the extracellular fluorescence, and $F_{in\_free}^{100\%}$ is the intracellular fluorescence correction factor at 10% power.

As indicated before, we used TMRM because unlike oxonol, TMRM allows measurement from single cells. The $V_m$ histograms for Days 2 and 8 for both 2-D and 3-D are shown in Figs. 2 and 3. On Day 2, the majority of cells in both 2-D and 3-D are in the range +20 to −20 mV. However, in 3-D cultures, the percentage of cells in the range −40 to −60 mV increased on Day 8 (9%) in comparison to Day 2 (5.6%). In contrast, 2-D cultures show a decrease on Day 8 (5.4%) from Day 2 (9.9%). A large percentage of cells examined did not develop a resting membrane potential of −55 mV, a figure reported by Sonnier et al. (2000). In contrast, the cells in our study exhibited a highly heterogeneous $V_m$ distribution. The $V_m$ results reported by Sonnier et al. (2000) were based on 10 cells and the measurements were conducted by the patch clamp technique. It is possible that cell selection was based on a morphology known to yield high resting membrane potentials. To ascertain the accuracy of our technique, we tested the relationship of dye concentration to gray levels by successively diluting TMRM stock solution (0.5 mM in 95% ethanol) to concentrations of 100,000, 10,000, 2000, 1000, 500, 250, 50, 5, and 0 nM. The average gray level was measured over the whole image. A corrected gray level reading was achieved by subtracting the background reading and data was fitted to a linear curve. This confirmed that the TMRM dye concentrations were linearly proportional to corrected gray level readings over the dye concentration range of 0–10,000 nM ($R^2 = 0.9962$).

### 3.3. Calcium dynamics

$Ca^{2+}$ ions function as a second messenger for a variety of cell processes. $Ca^{2+}$ causes contraction of muscle cells, neurotransmitters release, protein kinase activation, regulation of transcription, cell shape, and motility (Berridge et al., 1998). $Ca^{2+}$ ion channel dysfunction has been linked to diseases such as Alzheimer's, hypertension, angina, arrhythmia, etc. (Denyer et al., 1998). In order to establish 3-D cell preparation in collagen hydrogel as a functional biosensor we investigated the activity of voltage gated calcium channels.

Cells were depolarized with 50 mM high $K^+$ buffer on Days 2 and 8 of differentiation. For a cell at −50 mV, the high $K^+$ buffer resulted in a depolarization to +33.9 mV, well in the range of opening L- and N-Types channels. Fig. 4 shows a typical fluorescence intensity change for responsive and non-responsive cells. A cell that was considered responsive showed a 15% or higher calcium influx from the basal calcium levels (measured by change in relative fluorescent intensity values) upon addition of high $K^+$ buffer. Table 1 shows the percentage of responsive cells for 2-D and 3-D cultures. The maximum percentage of responsive cells in both 2-D and 3-D was only 25% which is not surprising based on the fraction of cells that developed a $V_m$ of −40 mV and higher on Day 8. The percentage of responsive cells in 2-D is 23% on Day 2 and 25% on Day 8, in contrast, Day 2 cells in 3-D did not show a high amount of response (13%) as compared to Day 8 (25%). This data supports the hypothesis (Mao and Kisaalita, 2004a) that calcium dynamics in 2-D may be exaggerated when compared to 3-D. The reason for this difference may be due to altered expression and regulation of VGCC in 2-D versus 3-D (Walsh and Parks, 2002). The average increase

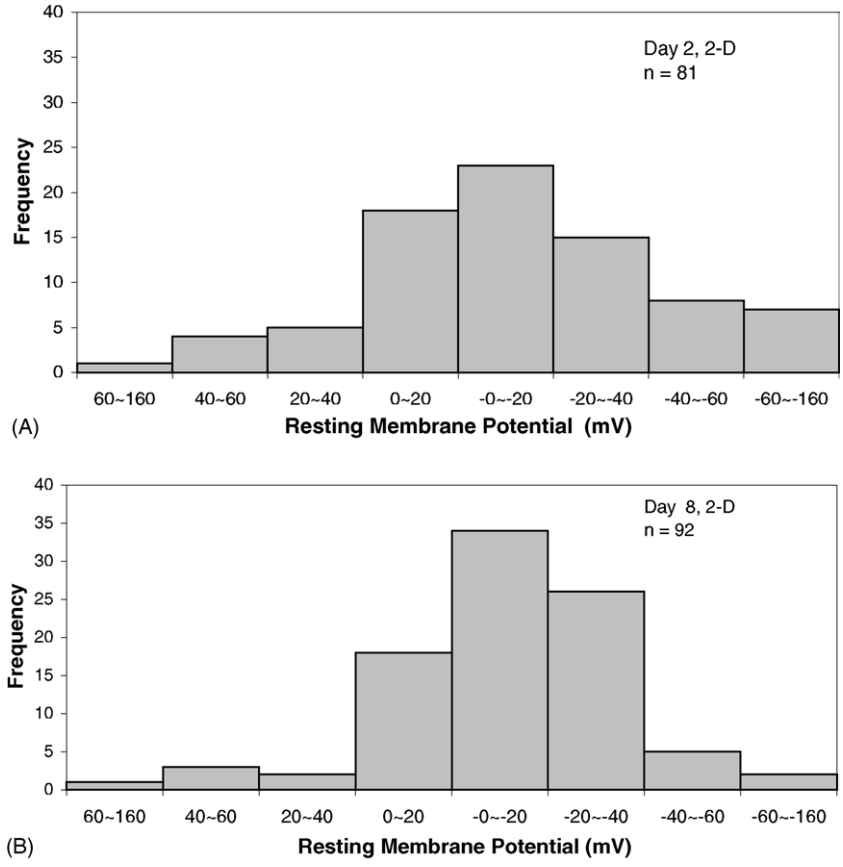


Fig. 2. A comparison of resting membrane potential histograms for 2-D, Day 2 (A) and Day 8 (B). Cells were differentiated using either dibutyryl cAMP and 5-bromodeoxyuridine or TPA and were loaded with 0.5 μM TMRM.

Table 1
Responsive cells[a]

| | 2-D (culture plates) | | 3-D (collagen gel) | |
|---|---|---|---|---|
| Number of days of culture in differentiation media | 2 | 8 | 2 | 8 |
| Total number of imaged cells | 78 | 88 | 76 | 92 |
| % of responsive cells | 23 | 25 | 13 | 25 |

[a] A cell was considered responsive if it exhibited a 15% or higher calcium influx from the basal calcium levels (measured by change in relative fluorescent intensity values) upon addition of high $K^+$ buffer (50 mM).

in calcium influx from basal levels also revealed differences between the 2-D and 3-D systems. In 2-D cultures, on Day 2, the calcium rise was 43% whereas on Day 8 it decreased to 30.5% ($p \leq 0.0251$). In 3-D cultures, the average increase was the same on Day 2 (45%) and Day 8 (50.9%) ($p \leq 0.3978$). Two-dimensional Day 2 calcium response was lower than 3-D Day 2 ($p \leq 0.0251$) and 2-D Day 8 calcium response was also lower than 3-D calcium response ($p \leq 0.0012$). The 3-D cultured cells responded with a higher calcium influx than 2-D cultured cells on both Days 2 and 8.

To further characterize the voltage gated calcium channel function in SH-SY5Y, Bay K 8644 was used to stimulate calcium release. Bay K 8644 is an agonist that selectively activates L-Type calcium channels (Reeve et al., 1995; Seward and Henderson, 1990). Table 2 shows the effect of Bay K 8644 on cells differentiated through Day 8 in 2-D and 3-D cultures. In comparison to 2-D, cells in 3-D responded better to both 5 μM (0% versus 4.3%) and 25 μM (1.6% versus 4.0%) of Bay K 8644. Overall, the low percentage of cells responding to Bay K 8644 confirmed that a majority of the L-Type voltage gated calcium channels in both cultures were not functional; alternatively, it has been reported

Table 2
Intracellular calcium increase in response to Bay K 8644

| | Bay K 8644 (μM) | |
|---|---|---|
| | 5 | 25 |
| 2-D (%) | | |
| Day 2 | 0.0 | 2.4 |
| Day 8 | 0.0 | 1.6 |
| 3-D (%) | | |
| Day 8 | 4.3 | 4.0 |

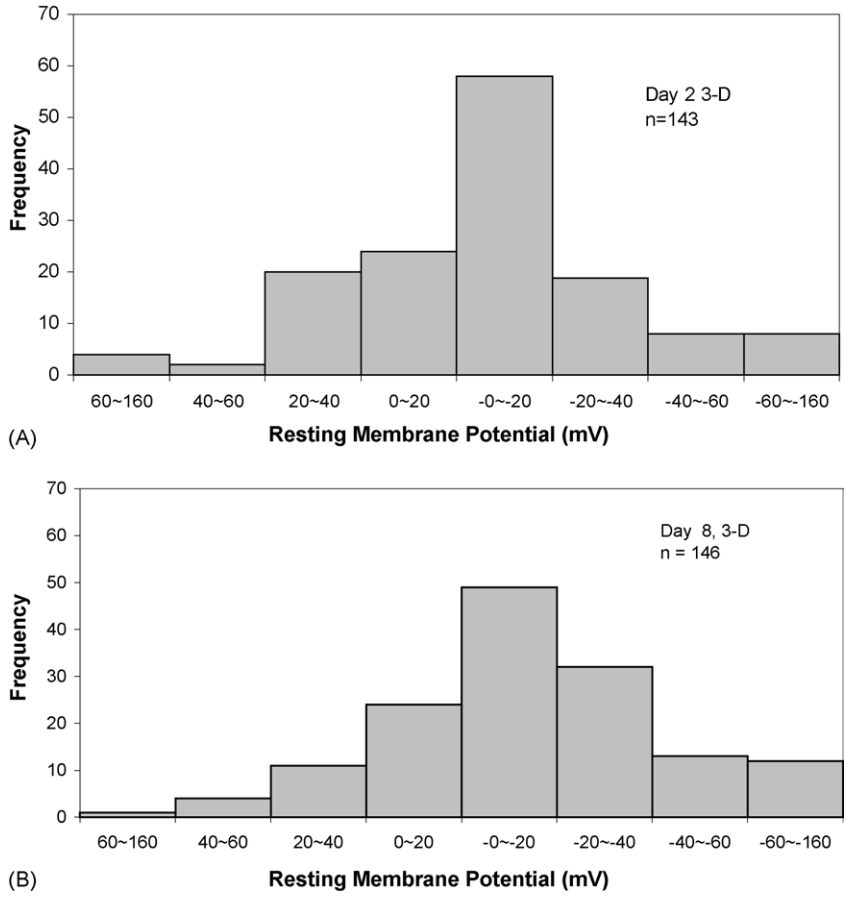


Fig. 3. A comparison of resting membrane potential histograms for 3-D, Day 2 (A) and Day 8 (B). Cells were differentiated using either dibutyryl cAMP and 5-bromodeoxyuridine or TPA and cells were loaded with 0.5 μM TMRM.

that voltage-dependent $Ca^{2+}$ entry occurs mainly through N-Type channels (Toselli et al., 1995). In order to confirm or rule out the possibility that VGCC were present and just non-functional, we conducted immunofluorescence experiments. There are five subtypes of voltage gated calcium channels (VGCC) in neuronal cells, which include L-, N-, P/Q-, R-, and T-Types (Hille, 1992). Numerous studies have confirmed the presence of L- and N-Types calcium channels in SH-SY5Y by using specific blockers and inducers (Morton et al., 1992; Reuveny and Narahashi, 1993; Seward and Henderson, 1990). However, the presence of P/Q-, R-, and T-Types of channels has not been documented in VGCC

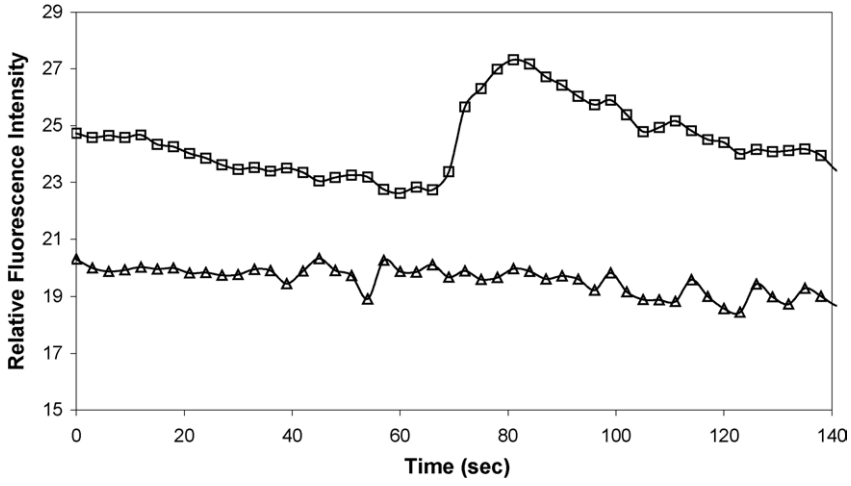


Fig. 4. Typical intracellular $Ca^{2+}$ change in response to depolarization with 50 mM $K^+$ in a responsive (□) and a non-responsive (△) cell.

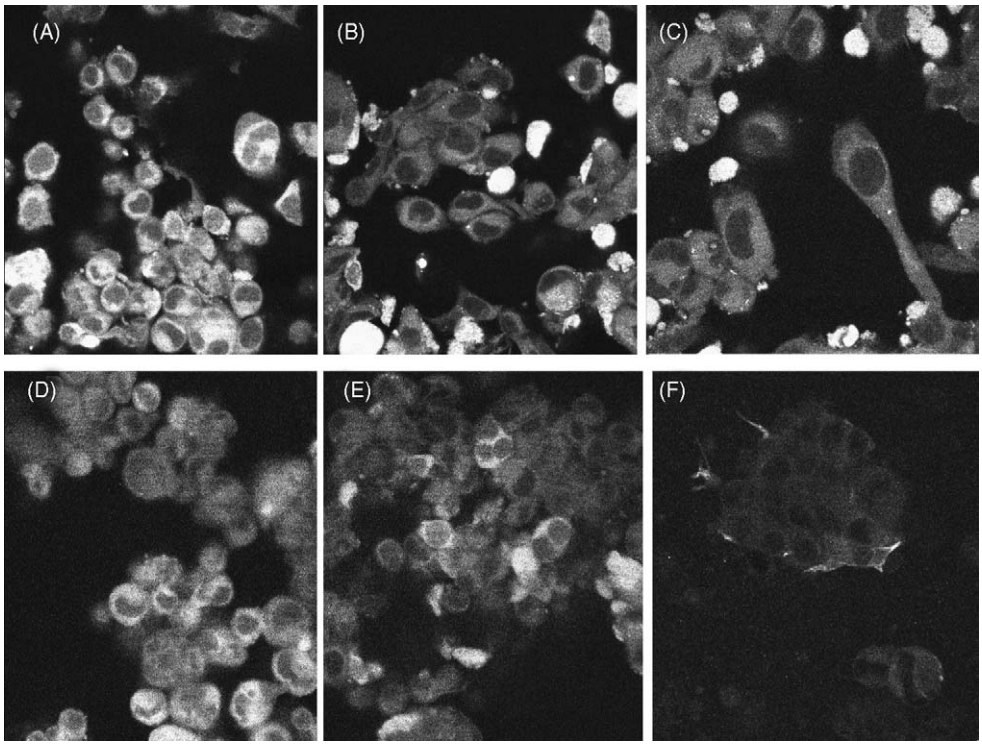


Fig. 5. Typical images of cells stained with BODIPY-FL verapamil for L-Type calcium channels. Top row is 2-D at Day 2 (A), Day 8 (B), and Day 13 (C) of culture in differentiation medium. Bottom row is 3-D at Day 2 (D), Day 8 (E), and Day 13 (F) of culture in differentiation medium.

studies in the SH-SY5Y cell line. Therefore, in our study, we chose to immunologically stain for the L- and N-Types VGCC and the results are presented below.

### 3.4. L- and N-Types staining

#### 3.4.1. L-Type channels

Fig. 5 shows typical images of cells stained with BODIPY-FL verapamil (L-Type) on Days 2, 8, and 13 in both 2-D and 3-D cultures. As shown in Fig. 6, the percentage of 2-D cells expressing L-Type channels progressively decreased from Day 2 (25.43%) to Day 8 (23.04%, $p \leq 0.0001$) and Day 13 (21.15%, $p \leq 0.0001$). In contrast, the percentage of 3-D cells expressing L-Type channels increased from 26.78 to 38.51% (Day 8, $p \leq 0.0001$) and 28.83% (Day 13, $p \leq 0.0001$). Increase in L-Type calcium channels with differentiation has been reported by others (Carbone et al., 1990; Usowicz et al., 1990; Kushmerick et al., 2001) in neuronal

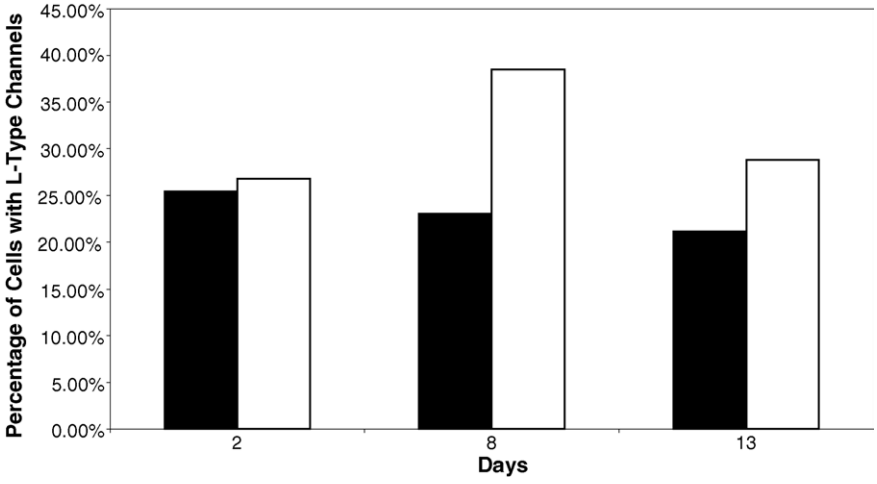


Fig. 6. A comparison of L-Type channel density in 2-D (■) and 3-D (□) culture cells. Day 2 number cells ($n$) = 44 and 64, mean fluorescence ($x$) = 223.5 and 209.5 arbitrary units, standard deviation (S.D.) = 17.0 and 18.7, for 2-D and 3-D, respectively. Day 8: $n$ = 59 and 58, $x$ = 254.0 and 330.6, S.D. = 2.3 and 19.8, for 2-D and 3-D, respectively. Day 13: $n$ = 33 and 47, $x$ = 197.9 and 204.7, S.D. = 31.9 and 33.6, for 2-D and 3-D, respectively.

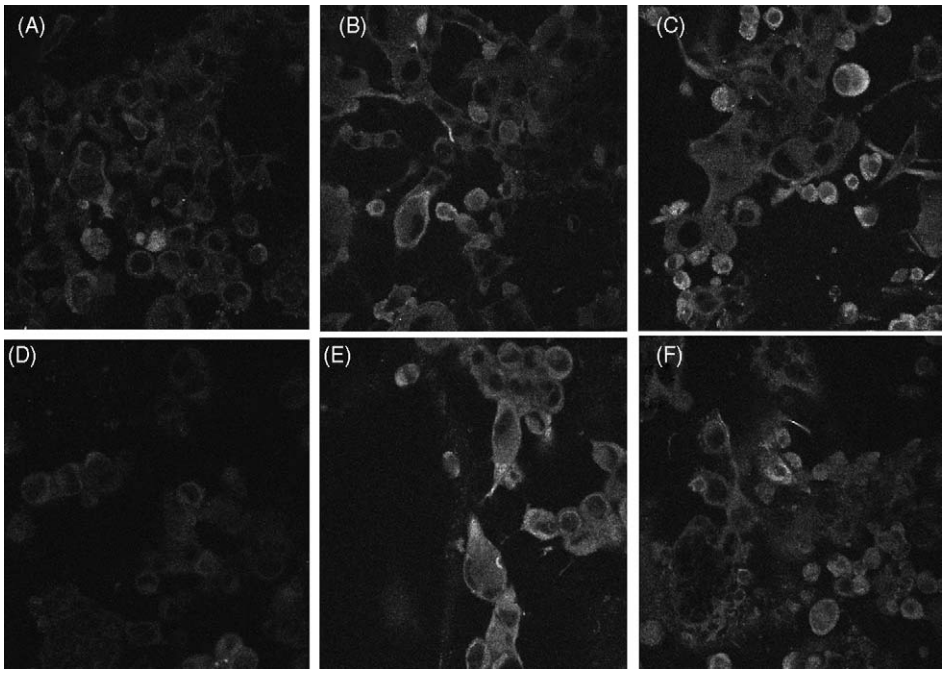


Fig. 7. Typical images of cells stained with anti-N Type channel antibody and Alexa 488-conjugated antibody for N-Type calcium channels. Top row is 2-D at Days 2 (A), 8 (B), and 13(C) of culture in differentiation medium. Bottom row is 3-D at Day 2 (D), Day 8 (E), and Day 13 (F) of culture in differentiation medium.

cells. The difference in L-Type channel development between 2-D and 3-D cultures points to the potential significance of culture format selection for cell-based biosensors. The L-Type channel expression levels based on staining results is inconsistent with Bay K 8644 channel activation results, strongly suggesting that either the majority of the channels were non-functional or could not be activated by Bay K 8644.

### *3.4.2. N-Type channels*

Fig. 7 shows typical images of cells stained with N-Type channel antibody on Days 2, 8, and 13 in both 2-D and 3-D cultures. As shown in Fig. 8, in 2-D, the percentage of cells expressing N-Type channels decreased from Day 2 (29.58%) to Day 8 (29.18%, $p \leq 0.0001$) and increased on Day 13 (29.32%, $p \leq 0.0001$) of differentiation. Although, the above differences in the means are low, significant differences were attributed to differences in cell numbers used for comparisons. In 3-D, the percentage of cells expressing N-Type channels increased from Day 2 (22.37%) to Day 8 (31.93%, $p \leq 0.0001$) and remained the same from Day 8 (31.93%) to Day 13 (31.84%, $p \leq 0.2584$). In 2-D, N-Type channel expression did not significantly change with differen-

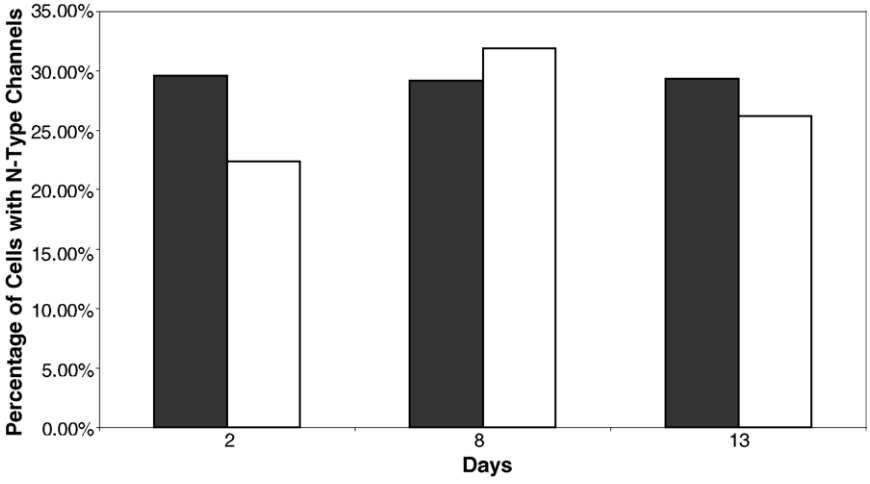


Fig. 8. A comparison of N-Type channel density in 2-D (■) and 3-D (□) culture cells. Day 2 number cells ($n$) = 21 and 17, mean fluorescence ($x$) = 84.5 and 75.7 arbitrary units, standard deviation (S.D.) = 20.7 and 16.0, for 2-D and 3-D, respectively. Day 8: $n$ = 75 and 53, $x$ = 104.3 and 99.3, S.D. = 24.8 and 20.1, for 2-D and 3-D, respectively. Day 13: $n$ = 73 and 70, $x$ = 126.9 and 93.8, S.D. = 30.3 and 37.0, for 2-D and 3-D, respectively.

tiation. As with L-Type channels, N-Type channel expression with differentiation in 3-D is consistent with previous studies (Toselli et al., 1995; Passafaro et al., 1992; Usowicz et al., 1990; Carbone et al., 1990).

Taken together, in 2-D cultures, both L- and N-Types channel expression decreased from Days 2 to 8 of differentiation. In 3-D, both types of channel expression increased with differentiation from Days 2 to 8. L- and N-Types channel expression on Day 13 for both 2-D and 3-D cultures presented inconsistent results, which has been attributed to the well-known slow increase in proportion of proliferating cells at long differentiation times. The general low functional channel expression renders use of SH-SY5Y cells in voltage gated ion channel cell-bases biosensor unfavorable. Primary or stem cells may provide better alternatives for future studies.

## 4. Conclusions

Recent studies have shown the contrast in cell behavior when evaluated in vivo and in vitro (Stoll et al., 2002; Miller et al., 2002) and this has led to the focus on the development of cell-based biosensors in three-dimensional formats that may more closely mimic the in vivo microenvironments. Our lab has previously studied IMR-32 cells in collagen hydrogel as a possibility for 3-D biosensor (Mao and Kisaalita, 2004a). However, the results were inconclusive, because IMR-32 failed to develop a resting membrane potential. In this study, we have used a human neuroblastoma cell line, SH-SY5Y to extend the previous study. The results from these experiments support the following conclusions:

(1) There was no statistically significant difference in SH-SY5Y proliferation rate between 2-D monolayer and 3-D collagen culture formats.
(2) A large percentage of cells (2-D culture, 91.30% and 3-D culture, 84.93%) did not develop resting membrane potential value equal to or lower than −40 mV; instead cells exhibited a heterogeneous resting membrane potential distribution.
(3) In response to high $K^+$ (50 mM) depolarization, 3-D cells were less responsive in comparison to 2-D cells, supporting the hypothesis that 2-D cell calcium dynamics may be exaggerated.
(4) L-Type expression levels based on staining results was inconsistent with Bay K 8644 channel activation results, strongly suggesting that either the majority of the channels were non-functional or could not be activated by Bay K 8644.

## References


Berridge, M., Bootman, M., Lipp, P., 1998. Calcium-a life and death signal. Nature 395, 645–648.

Cukierman, E., Pankov, R., Stevens, D., Yamada, K., 2001. Taking cell-matrix adhesions to the third dimension. Science 294, 1708–1712.

Cukierman, E., Pankov, R., Yamada, K., 2002. Cell interactions with three-dimensional matrices. Curr. Opin. Cell Biol. 14, 633–639.

Carbone, E., Sher, E., Clementi, F., 1990. $Ca^{2+}$ currents in human neuroblastoma IMR32 cells: kinetics, permeability, and pharmacology. Pflugers Arch. 416, 170–179.

Deng, Y., Zhao, K., Zhang, X., Hu, P., Chen, G., 2002. Study on the three-dimensional proliferation of rabbit articular cartilage-derived chondrocytes on polydroxyalkanoate scaffolds. Biomaterials 23 (20), 4049–4056.

Denyer, J., Worley, J., Cox, B., Allenby, G., Banks, M., 1998. HTS approaches to voltage-gated ion channel drug discovery. Drug Discov. Today 3, 323–332.

Durick, K., Negulescu, P., 2001. Cellular biosensors for drug discovery. Biosens. Bioelectron. 6, 587–592.

Ehrenberg, B., Montana, V., Wei, M., Wuskell, J., Loew, L., 1988. Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. Biophys. J. 53, 785–794.

Friedl, P., Brocker, E., 2000. The biology of cell locomotion within three-dimensional extracellular matrix. Cell. Mol. Life Sci. 57, 41–64.

Gonzalez, J., Oades, K., Laychkis, Y., Harootunian, A., Negulescu, P., 1999. Cell-based assays and instrumentation for screening ion-channel targets. Drug Discov. Today 4, 431–439.

Hille, B., 1992. Ionic Channels of Excitable Membranes. Sinauer, Sutherland, MA.

Ignatius, A., Blessing, H., Leidert, A., Schmidt, C., Neidlinger-Wilke, C., Kaspar, D., Friemert, B., Claes, L., 2005. Tissue engineering of bone: effects of mechanical strain on osteoblastic cells in type I collagen matrices. Biomaterials 26 (3), 311–318.

Jessup, J., Goodwin, T., Spaulding, G., 1993. Prospects for use of microgravity-based bioreactors to study three-dimensional host–tumor interactions in human neoplasia. J. Cell. Biochem. 51 (3), 290–300.

Kisaalita, W., Bowen, J., 1997. Effect of medium serum concentration on N1-E115 membrane potential development. In Vitro Cell. Dev. Biol. Anim. 33 (3), 152–155.

Kleinman, H., Klebe, R., Martin, G., 1981. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88, 473–485.

Kushmerick, C., Romano-Silva, M., Gomez, M., Prado, M., 2001. Changes in $Ca^{2+}$ channel expression upon differentiation of SN56 cholinergic cells. Brain Res. 916, 199–210.

Loew, L., 1998. Measuring membrane potential in single cells with confocal microscopy. In: Celis, J.E. (Ed.), Cell Biology, A Laboratory Handbook, vol. 3. Academic Press, San Diego, CA, pp. 375–379.

Mao, C., Kisaalita, W., 2004a. Characterization of 3-D collagen hydrogels for functional cell-based biosensing. Biosens. Bioelectron. 9, 1075–1088.

Mao, C., Kisaalita, W.S., 2004b. Determination of resting membrane potential of individual neuroblastoma cells (IMR-32) using a potentiometric dye (TMRM) and confocal microscopy. J. Fluorescence 14 (6), 739–743.

Matthews, J., Wnek, G., Simpson, D., Bowlin, G., 2002. Electrospinning of collagen nanofibers. Biomacromolecules 3 (2), 232–238.

Miller, M., Wei, S., Parker, I., Cahalan, M., 2002. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science 296, 1869–1873.

Morton, A.J., Hammond, C., Mason, W., Henderson, G., 1992. Characterization of the L- and N-type calcium channels in differentiated SH-SY5Y neuroblastoma cells: calcium imaging and single channel recording. Mol. Brain Res. 13, 53–61.

Nakamura, Y., Sagara, T., Seki, K., Hirano, S., Nishida, T., 2003. Permissive effect of fibronectin on collagen gel contraction mediated by bovine trabecular meshwork cells. Invest. Ophthalmol. Vis. Sci. 44 (10), 4331–4336.

O'Connor, S., Amdreadis, J., Shaffer, K., Ma, W., Pancrazio, J., Stenger, D., 2000a. Immobilization of neural cells in three-dimensional matri-

ces for biosensor applications. Biosens. Bioelectron. 14 (10–11), 871–881.

O'Connor, S., Stenger, D., Shaffer, K., Maric, D., Barker, J., Ma, W., 2000b. Primary neural precursor cell expansion, differentiation and cytosolic $Ca^{2+}$ response in three-dimensional collagen gel. J. Neurosci. Methods 102, 187–195.

Passafaro, M., Clementi, F., Sher, E., 1992. Metabolism of ω-conotoxin-sensitive voltage-operated calcium channels in human neuroblastoma cells: modulation by cell differentiation and anti-channel antibodies. J. Neurosci. 12 (9), 3372–3379.

Powers, M., Domansky, K., Kaazempur-Mofrad, M., Kalezi, A., Capitano, A., Upadhyya, A., Kurzawski, P., Wack, K., Stolze, D., Kamm, R., Griffith, L., 2002. A microfabricated array bioreactor for perfused 3D liver culture. Biotechnol. Bioeng. 78 (3), 257–269.

Reeve, H., Vaughan, P., Peers, C., 1995. Enhancement of $Ca^{2+}$ channel currents in human neuroblastoma (SH-SY5Y) cells by phorbol esters with and without activation of protein kinase C. Eur. J. Physiol. 429, 729–737.

Reuveny, E., Narahashi, T., 1993. Two types of high voltage-activated calcium channels in SH-SY5Y human neuroblastoma cells. Brain Res. 603, 64–73.

Rao, R., Kisaalita, W., 2001. Biochemical and electrophysiological differentiation profile of a human neuroblastoma (IMR-32) cell line. In Vitro Cell. Dev. Biol. Anim. 38 (8), 450–456.

Senoo, H., Imai, K., Sato, M., Kojima, N., Miura, M., Hata, R., 1996. Three-dimensional structure of extracellular matrix reversibly regulates morphology, proliferation, and collagen metabolism of perisinusoidal stellate cells (vitamin A-storing cells). Cell Biol. Int. 20 (7), 501–512.

Seward, E., Henderson, G., 1990. Characterization of two components of the N-like, high-threshold-activated calcium channel current in differentiated SH-SY5Y cells. Pflugers Arch. 417, 223–230.

Sherwood, J., Riley, S., Palazzolo, R., Brown, S., Monkhouse, D., Coates, M., Griffith, L., Landeen, L., Ratcliffe, A., 2002. A three-dimensional osteochondral composite scaffold for articular cartilage repair. Biomaterials 23 (24), 4739–4751.

Sonnier, H., Kolomytkin, O., Marino, A., 2000. Resting potential of excitable neuroblastoma cells in weak magnetic fields. Cell. Mol. Life Sci. 57, 514–520.

Stoll, S., Delon, J., Brotz, T., Germain, R., 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science 296, 1873–1876.

Toselli, M., Perin, P., Taglietti, V., 1995. Muscarine inhibits ω-conotoxin-sensitive calcium channels in a voltage and time-dependent mode in the human neuroblastoma cell line SH-SY5Y. J. Neurophysiol. 74, 1730–1741.

Traill, K., Bock, G., Winter, U., Hilchenbach, M., Jurgens, G., Wick, G., 1986. Simple method for comparing large numbers of flow cytometry histograms exemplified by analysis of the CD4 (T4) antigen and LDL receptor on human peripheral blood lymphocytes. J. Histochem. Cytochem. 34 (9), 1217–1221.

Usowicz, M., Porzig, H., Becker, C., Reuter, H., 1990. Differential expression by nerve growth factor of two types of $Ca^{2+}$ channels in rat phaeochromocytoma cell lines. J. Physiol. 426, 95–116.

Walsh, K., Parks, G., 2002. Changes in cardiac myocyte morphology alter the properties of voltage-gated ion channels. Cardiovasc. Res. 55, 64–75.