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Fluorescent Detection of Merlin-Deficient Schwann Cells and Primary Human Vestibular Schwannoma Cells Using Sodium Fluorescein

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Abstract

Hypothesis—Merlin-deficient Schwann cells (MD-SC) and primary human vestibular schwannoma (VS) cells exhibit selective uptake of sodium-fluorescein (SF), allowing for fluorescent detection and improved visualization of tumor cells, when compared to Schwann cells (SC).

Background—SF is a fluorescent compound used for fluorescence-guided resection of gliomas. The utility of SF for VS surgery has not been assessed.

Methods—Mouse MD-SCs and rat SCs were cultured on 96-well plates at different cell densities and treated with SF at several drug concentrations and durations. Relative fluorescence units (RFU) were measured using a fluorometer to determine optimal treatment parameters *in vitro*. Subsequently, a four-point Likert scale for fluorescence visualization of pelleted cells was created and validated. Blinded observers rated SF-treated primary human VS and SC cultures, which were developed from de-identified specimens obtained from live and cadaveric donors, respectively.

Results—In contrast to SCs that showed low levels of fluorescence, MD-SCs demonstrated dose-dependent increases in RFUs when treated with incremental dosages of SF as well as longer treatment and fluorescent excitation times. In addition, RFUs were higher at greater MD-SC densities. The Likert scale for fluorescence visualization was validated using 9 blinded observers and there were excellent inter- and intra-rater reliabilities (intra-class coefficients of 0.989 and >0.858, respectively). Using the Likert scale, human VS treated with SF received higher scores than human SCs ($p < 0.001$).

Conclusion—Mouse MD-SC and human VS cells demonstrate preferential uptake of SF when compared to normal primary SCs. Observers detected differences in fluorescence using the validated Likert scale. Further investigations into the utility of SF-guidance in VS surgery is warranted.

Introduction

The use of sodium fluorescein (SF) as a fluorescent dye to distinguish *in vivo* neoplastic brain tissue from surrounding healthy tissue dates back as early as the 1940s.¹ In a report by Moore, a majority of brain and spinal cord tumors demonstrated high levels of fluorescence¹. This early experience relied on tumor visualization under ultraviolet light, pre-dating current sophisticated techniques using microscope-assisted visualization and dedicated fluorescent filters.

Over recent years, the neurosurgical literature has extensively reported on favorable outcomes using this technique for intraoperative visualization of parenchymal tumors, such as high-grade gliomas. An intravenous (IV) route of administration is employed in this technique and it is believed that SF exerts a non-specific mechanism of tumor targeting by exploiting a leaky brain blood barrier (BBB) in areas of high tumor cell density.² Hence, abnormal microvasculature in these lesions can allow for better visualization of tumor margins. This maximizes chances for a complete resection while minimizing collateral injury to surrounding tissue. Indeed, recent studies have confirmed this hypothesis both *in vitro* and *in vivo*.^{3–8}

Cultures of human astrocytes and malignant U251 glioma cells have demonstrated minimal to no uptake of SF.³ Diaz *et al.* further verified this using a mouse model of intracranial U87 malignant glioma xenografts as well as a genetically-induced glioma mouse model. The conclusion of these experiments was that IV SF leaks through a tumor-damaged BBB into the extracellular space, thereby demarcating the margins of the lesion. This process occurs without any selective uptake by tumor cells. Therefore, false negative results can occur at regions of tumor necrosis, which lack adequate microvasculature, as well as areas with lower tumor densities and normal BBB.³ Nevertheless, the sensitivity and specificity of SF fluorescence to identify gliomas are in the range of 80–96 and 79–90 percent, respectively.^{3,8,9} This has led to significant improvements in rates of gross total resection with this particular disease. Whether this technique ultimately translates to a survival advantage for glioma patients remains to be determined with larger cohort studies.³

Unlike malignant intraparenchymal tumors, intracranial schwannomas found along various skull base subsites pose a different set of challenges for surgeons. Gliomas are notorious for poorly defined margins while skull base schwannomas are often well-encapsulated benign tumors. Depending on their location, when schwannomas grow, they exert significant distortive forces on the healthy surrounding neurovascular structures, such as the brain and cranial nerves. Therefore, as is often the case with surgery for Neurofibromatosis Type II (NF2)-associated and large sporadic vestibular schwannomas (VS), identifying the proper tumor-nerve and tumor-brain interface becomes a critical and challenging portion of the

case. To our knowledge, the concept of fluorescence-guided surgery as exemplified with the use of SF has not been studied extensively in the setting of VS surgery.

Recent studies by da Silva *et al.* promoted the idea of SF-guided skull base surgery.^{10,11} In their initial study assessing three meningiomas, one craniopharyngioma, one pituitary adenoma, and one VS, they demonstrated that tumor enhancement could be detected following IV SF administration using standard white light microscopic visualization and photography.¹⁰ However, the sample size was small and fluorescent excitation and quantification of emissions were not utilized.^{10,11}

Fluorescent detection of benign skull base tumors using IV SF may rely on different mechanisms of action than that found for intraparenchymal malignant gliomas. Considering the relatively low cost of SF and mild side effect profile, it is worth considering new applications for its use, such as differentiating tumor from adjacent nerve and brain in VS surgery.² Because glial cells, such as Schwann cells (SC) and oligodendrocytes, ensheath nerve fibers, identifying fluorescent compounds that preferentially enter VS cells compared to normal glial cells can potentially aid in identifying the tumor-nerve and tumor-brain interfaces. This study investigates the differences in fluorescent detection of merlin-deficient Schwann cells (MD-SC) and primary human VS cell compared to normal SCs following treatment with SF *in vitro*.

Methods

Cell Lines and Primary Human VS Cells

Primary rat Schwann cells (RSC) and human Schwann cells (HSC) were isolated, expanded and cryopreserved as described previously.^{12,13} RSCs were isolated from sciatic nerves of neonatal rats through a University of Miami Institutional Animal Care and Use Committee (IACUC) approved protocol.¹² HSCs were derived from de-identified normal peripheral nerve specimens obtained from cadaveric donors through the Life Alliance Organ Recovery Agency of the University of Miami Miller School of Medicine, using an Institutional Review Board (IRB) exempt protocol.¹³ RSC and HSC were cultured on 0.01% poly-L-ornithine (Sigma Aldrich) and laminin (5µg/ml; ThermoFisher) coated culture flasks in mitogenic media, consisting of Dulbecco's Modified Eagle Medium (GIBCO), 10% heat inactivated fetal bovine serum (FBS; Seradigm), forskolin (2µM; Sigma Aldrich), heregulin (2.25µM; Sigma Aldrich) and 1% penicillin-streptomycin at 37 °C, 5% CO₂.

Mouse MD-SCs (FV-MNMSC-032910 aka MTC-10+luciferase) were prepared according to reported methods.¹⁴ Under a University of Central Florida IACUC approved protocol, sciatic nerves were harvested from homozygous *Nf2^{flox2/flox2}* mice that were 3–6 weeks old. Primary *Nf2^{flox2/flox2}* SCs were isolated and then purified using differential laminin adhesion. Cells were transduced overnight with Adeno-Cre virus at 2nd passage and deletion of *Nf2* exon 2 and merlin protein were confirmed with polymerase chain reaction and western blot analysis, respectively.¹⁴ MD-SCs were cultured in 0.01% poly-L-ornithine and laminin (5µg/ml) coated culture flasks with N2 media consisting of DMEM/F12 (ThermoFisher), 1% N2 supplement (ThermoFisher), and 1% penicillin-streptomycin (Sigma Aldrich) at 37 °C, 5% CO₂.

Primary human VS cells were cultured from de-identified fresh VS chunks obtained through the Tissue Bank Core Facility at the University of Miami Miller School of Medicine through an IRB exempt protocol. Fresh VS tumor was divided into 1×1×1 mm pieces and enzymatically dissociated and cultured on 0.1% poly-L-lysine (Sigma Aldrich) and laminin (25µg/ml) coated plates in SC medium (Sciencell), consisting of 500 ml of basal medium, 5% FBS, 1% SC growth supplement, and 1% penicillin-streptomycin, at 37 °C, 5% CO₂, as previously described.^{15,16}

Sodium Fluorescein Assays

MD-SCs and RSCs were cultured on 96-well plates that were pre-coated with 0.01% poly-L-ornithine and laminin (5µg/ml) at several cell densities (0–100,000 cells per well) for 24 hours in their respective culture media at 37 °C, 5% CO₂. After 24 hours, cells were treated with various concentrations of SF (0–1250 µM) in the above referenced media for various treatment durations (0–6 hours). Plates were washed three times with Hank's balanced salt solution (HBSS; Sigma Aldrich) and fluorescence was measured in relative fluorescence units (RFU) using the GloMax Discover (Promega) at the following settings: excitation of 475 nm and emissions in the range of 500–550 nm. RFUs were recorded at 0 minutes and after 10, 20, and 30 minutes of continuous blue light excitation. Each experimental condition was analyzed using 4–5 replicate samples. The optimal *in vitro* parameters for SF concentration and treatment duration to maximize fluorescent detection were determined and utilized for subsequent studies involving direct visualization of fluorescent cells.

Validation of Likert Scale for Direct Visualization of Fluorescent Cells

To test the ability of observers to visually detect the degree of fluorescence, a four-point Likert scale was created. MD-SCs and RSCs were trypsinized and treated with SF (1000µM) or vehicle (0.05% dimethyl sulfoxide [DMSO]) as a suspension at 37 °C, 5% CO₂, for 3 hours. Subsequently, cells were washed three times with HBSS. To simulate the 3-dimensional nature of tissue and tumor, cells were immediately pelleted in clear polymerase chain reaction (PCR) tubes at various densities (0 to 1 million cells per tube). PCR tubes containing 5 µL of HBSS were utilized as negative controls. Cells were exposed to continuous blue light excitation for 30 minutes at room temperature. Nine blinded raters were asked to rate the fluorescence of pelleted cells using the Likert scale. To validate the Likert scale, inter-rater and intra-rater reliabilities were calculated.

Direct Visualization of Fluorescent HSC and Human VS Cells

Two different HSC cultures and six independent primary human VS cell cultures were treated with SF (1000µM) or vehicle (0.05% dimethyl sulfoxide [DMSO]) as a cell suspension for 3 hours at 37 °C and 5% CO₂. Cells were subsequently washed three times with HBSS and 500,000 cells of each sample were pelleted in PCR tubes. Blank PCR tubes were used as negative controls. Cells were exposed to continuous blue light excitation for 30 minutes at room temperature. Seven to ten blinded observers were asked to rate the fluorescence using the validated Likert scale.

Statistical Analysis

Analysis of Variance using Tukey Honestly Significant Difference (HSD) was utilized to compare differences among conditions. Significance was set at p -value < 0.05 . To validate the Likert scale, intra-class coefficients were calculated to determine inter- and intra-rater reliabilities. Excellent reliability was considered to be intra-class coefficients > 0.8 .

Results

Fluorescence by Drug Concentration and Duration of Blue Light Excitation

Mouse MD-SCs and RSCs were plated at a cell density of 50,000 cells per well and were treated with various concentrations of SF for 3 hours *in vitro*. Relative fluorescence units (RFU) were measured after 0, 10, 20, and 30 minutes of blue light excitation. RSCs demonstrated stable levels of RFUs at all concentrations of SF tested and all time points of blue light excitation ($p > 0.05$; Figure 1A). In contrast, MD-SCs treated with higher dosages of SF demonstrated dose-dependent increases in RFUs. Fluorescence measurements were also higher with longer blue light excitation times, with 30 minutes of excitation producing the greatest levels of RFUs (Figure 1B). Following 30 minutes of blue light excitation, MD-SCs produced significantly higher levels of RFUs than RSCs at 250, 500, 750, 1000, and 1250 μM concentrations of SF ($p = 0.015$, $p = 0.024$, $p = 0.006$, $p = 0.002$, $p = 0.0001$, respectively). There was a plateau in RFUs at $\sim 1000 \mu\text{M}$ of SF (Figure 1C); therefore, this concentration was utilized for subsequent fluorescence testing.

Fluorescence by Cell Density

MD-SCs and RSCs were cultured on 96-well plates at cell densities ranging from 0 to 100,000 cells per well and treated with 1000 μM SF for 3 hours. Subsequently, RFUs were measured following 30 minutes of blue light excitation. MD-SCs demonstrated significantly higher levels of relative fluorescence units (RFU) than RSCs at cell densities $> 40,000$ cells per well. At $\sim 80,000$ cells per well, there was a plateau in RFU measurements (Figure 2A), representing the approximate cell density at which the entire two-dimensional surface area of the well is saturated with a monolayer of cells. Using 80,000 cells per well, MD-SCs and RSCs were treated with 1000 μM SF for 0 to 6 hours and RFUs were determined after 30 minutes of blue light excitation. There were significantly higher levels of RFUs in MD-SCs at treatment durations ranging between 3 to 6 hours, compared to RSCs (Figure 2B).

Validation of Likert Scale for Fluorescence Visualization

A four-pointed Likert scale for fluorescence visualization of pelleted cells in PCR tubes was created (Figure 3A). MD-SCs and RSCs were treated with 1000 μM SF or vehicle (0.05% DMSO) for 3 hours and subsequently pelleted into PCR tubes in different amounts ranging from 0 to 1 million cells per tube. Following 30 minutes of blue light excitation, nine blinded observers were asked to rate the intensity of the green fluorescence emitted from each tube under blue light. The mean Likert scores for MD-SCs treated with SF were significantly higher than scores obtained from RSCs treated with SF and untreated MD-SCs and RSCs (Figure 3B). The inter-rater reliability was excellent, with an intraclass coefficient

[ICC] of 0.989 [95% Confidence Interval of 0.983–0.993]. The intra-rater reliabilities were excellent for all raters (ICC >0.858), thereby validating the Likert scale.

Likert Scores for Human Schwann Cells and Vestibular Schwannoma Cells

HSCs and human VS cells were treated with 1000 μ M of SF for 3 hours and 500,000 cells were pelleted in PCR tubes and exposed to blue light for 30 minutes. Seven to ten blinded observers rated the fluorescent intensities of the pelleted cells under blue light excitation. Representative images of conditions are displayed in Figure 4A. The overall Likert Scores for SF-treated HSC and human VS cells were significantly higher than the control condition ($p=0.0006$ and $p=5.36\times 10^{-13}$, respectively). Furthermore, the overall Likert scores for SF-treated human VS were significantly higher than that for HSCs treated with SF ($p=5.36\times 10^{-13}$; Figure 4B). When analyzing individual samples, there were no significant differences in Likert scores between HSC samples or between human VS samples ($p>0.05$). However, when analyzing multiple comparisons between individual human VS and HSC samples, significant differences were noted between both groups (Figure 4C).

Discussion

SF is a biocompatible fluorescent dye routinely used in the fields of medicine for various applications. SF absorbs light in the wavelength spectrum of 460–500 nm and emits visible fluorescent light in the spectrum of 549–690 nm. In neurosurgery, investigations utilizing SF in fluorescence-guided resection of glioma suggest that the primary mechanism of uptake by the tumor is through a leaky BBB located in areas of high tumor density that demonstrate abnormal microvasculature.^{2,3} However, research involving topical SF in ophthalmology suggests that uptake of SF is also cell type-specific. There are sub-populations of cells that internalize much higher levels of SF and enhance significantly brighter than other cell types.¹⁷

Our *in vitro* investigations support a preferential cell-specific uptake of SF in MD-SCs, compared to normal RSCs. In MD-SCs, the fluorescence intensities were also dependent on concentration of SF, duration of treatment, cell density, and total time of blue light excitation. Although these quantitative RFU measurements are informative, the ability of neurologists and neurosurgeons to assess fluorescence in more three-dimensional models would be more applicable to patient care. Therefore, we created a validated Likert Scale for fluorescence visualization and asked blinded observers to detect differences in fluorescence in pelleted cells, which has more clinical relevance than two-dimensional plate models. Although fluorescent measurements of pelleted cells using a standard fluorometer for plated cells is not possible, the RFU measurements obtained from 96-well plate studies can be extrapolated to estimate the degree of fluorescent intensities of pelleted cells.

The findings of these studies demonstrate that blinded observers rated the fluorescence intensities of MD-SCs significantly higher than that seen in normal RSCs. In addition, for MD-SCs, we demonstrated that raters were able to detect differences in cell density based on emitted fluorescence during blue light excitation. These encouraging results supported further investigations into the utility of SF in detecting human schwannoma cells and

differentiating them from normal human SCs that ensheath neuronal fibers of adjacent peripheral nerves.

In this pilot study, we demonstrated that primary human VS cultures obtained from 6 independent donors have selective uptake of SF, in comparison to two HSC cultures obtained from 2 separate donors. Blinded observers were able to distinguish differences in fluorescence between the human VS and HSC groups using our validated Likert scale. Cell senescence occurs with cell expansion of primary human VS and HSC cultures; therefore, we limited experiments to the first five passages of primary cells. Because of limitations in the quantity of cells for testing, primary human cells were not plated on 96-well plates and RFUs were not measured. Nevertheless, these findings are highly encouraging and warrant additional investigations in preclinical and clinical settings.

Although there are inherent limitations using *in vitro* models, we show a robust pattern of fluorescence following various SF treatment conditions with MD-SCs and several primary human VS cultures. The mechanisms of cell-specific uptake were not evaluated in depth nor were neuronal and oligodendrocyte cultures utilized, representing future directions in optimizing fluorescence-guided surgery. Furthermore, the ability of SF to penetrate the BBB in areas of abnormal tumor microvasculature or compressed cerebellum or brainstem cannot be assessed in this *in vitro* study and requires immunohistochemical methods using *in vivo* model systems.

The optimal SF concentration and treatment duration, elicited from our *in vitro* experiments, cannot be fully extrapolated to make conclusions regarding SF dosing strategies in an *in vivo* model or in clinical trials; drug titration studies will be necessary to determine these parameters. Fortunately, *in vivo* models for schwannoma and VS exist and the variables affecting SF uptake and clearance can be investigated in xenograft and allograft models of VS as well as genetic models of NF2.^{16,18–20}

In addition, direct visualization of fluorescence was evaluated under blue light excitation; it is possible that when using surgical microscopes with appropriate filters for SF fluorescence, the enhancing tumor and separation from normal adjacent structures may be better appreciated. Lastly, because VS tumors were de-identified, information on clinical presentation, radiographic findings, and clinical pathology could not be obtained from the institutional medical records. However, regardless of this lack of clinically relevant information, all human VS cells demonstrated high levels of SF uptake.

When entertaining the possibility of introducing SF as an adjunct to an intracranial skull base procedure, physicians must also consider possible risks such as induction of seizures and anaphylaxis when used intrathecal or intravenous, respectively.^{20–22} This is particularly important in the setting of surgery for VS, which is considered a benign disease with frequent possibilities for non-surgical treatment. Fortunately, reports of adverse events have been limited to very few case reports in the literature, giving SF a relatively suitable safety profile for this application.^{20–23}

Conclusion

Similar to its use in neurosurgical procedures for resection of intraparenchymal gliomas, SF may have great implications for use in VS surgery, where the planes between tumor and adjacent nerve and brain can be difficult to establish. SF-guided VS surgery can potentially improve the chance of complete surgical resection and prevent devastating functional morbidities, such as facial nerve paralysis. The current study is the first to demonstrate that SF can be used to preferentially target and enhance human VS cells when compared to HSCs *in vitro*, and this difference is visible to unbiased observers without the use of a microscope or special equipment. Further *in vitro* and *in vivo* studies are necessary to understand the mechanisms of interaction between SF and human VS and HSCs with the ultimate goal of applying its use in human VS surgery to improve both extirpative and functional outcomes.

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Figure 1. Relative Fluorescence Units by Drug Concentration and Duration of Blue Light Excitation

[A-B] Mouse merlin-deficient Schwann cells (MD-SCs) demonstrated a dose-dependent increase in RFUs, while rat Schwann cells (RSCs) demonstrated overall stable RFUs at all blue light excitation durations. In MD-SCs, there is a plateau in RFUs at $\sim 1000\mu\text{M}$ of sodium fluorescein. [C] After 30 minutes of blue light excitation, MD-SCs demonstrated higher RFU values than RSCs at multiple concentrations of sodium fluorescein. Markers represent averages. Error bars denote standard deviation. * $p < 0.05$. ** $p < 0.01$.

Figure 2. Relative Fluorescence Units by Cell Density after 30 Minutes of Blue Light Excitation

[A] Mouse merlin-deficient Schwann cells (MD-SC) demonstrated significantly higher levels of relative fluorescence units (RFU) than rat Schwann cells (RSCs) at cell densities 40,000 cells per well. There was a plateau in RFUs, starting at ~80,000 cells per well. [B] When evaluating treatment duration following 30 minutes of blue light excitation, RFUs increased significantly in MD-SCs at 3-hour treatment durations, compared to RSCs. There were no differences between RFUs at 3–6-hour treatment durations. Bars represent averages. Error bars denote standard deviation. * $p < 0.05$. ** $p < 0.01$. NS, not significant.

Figure 3. Likert Scale and Scores for Fluorescence Visualization

[A] A four-point Likert scale was created for visualizing green fluorescence emitted from mouse merlin-deficient Schwann cells (MD-SC) and normal rat Schwann cells (RSCs). [B] Nine raters were asked to rate the intensity of green fluorescence (using the Likert scale) in MD-SC and RSC pellets treated with and without sodium fluorescein (1000 μ M) for 3 hours. There was a significant difference in the mean Likert scores for MD-SC treated with sodium fluorescein, compared to the remaining three groups. The inter-rater reliability was excellent (intraclass coefficient [ICC] of 0.989; 95% Confidence Interval [0.983–0.993]). The intra-rater reliabilities were excellent for all raters (ICC >0.858). Markers represent mean scores. Error bars represent standard deviation. **p<0.01.

Figure 4. Likert Scores for Human Schwann Cells and Vestibular Schwannoma Cells

[A] Representative images of an empty PCR tubes and PCR tubes containing 500,000 pelleted human Schwann cells (HSCs) and human VS cells following treatment with sodium fluorescein (1000 μ M) for 3 hours. Tubes were exposed to 30 minutes of blue light excitation. **[B]** Overall Likert Scores for HSCs and human VS cells were significantly higher than the control condition. The overall scores for 6 human VS were also significantly higher than the 2 HSC samples derived from two different organ donors (Schwann 116 and 216). **[C]** Likert scores for individual human VS and HSC were consistent within each group. However, the scores for all human VS samples were significantly higher than ratings for both HSC conditions. Bars represent averages. Error bars represent standard deviation. * $p < 0.05$. ** $p < 0.01$. NS, not significant.