

Role of Gap Junctions in Glioblastoma Multiforme Brain Tumors

By

Muna Oli

Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Bachelors of Science

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Abstract

Cancer is the second leading cause of death in USA, yet relatively little is known about how a collection of cancer cells form primary tumors. Recently, scientists have started studying tumors as a complex adaptive system in which a tumor is composed of heterogeneous collection of individual cancer cells that interact with neighboring cells and adapt to their local environments. A key premise of this thinking is that cancer cells are able to interact and communicate with each other via gap junctions, and that disruption of cell-to-cell communication should adversely affect tumors as a complex system. However, whether or not and to what extent Glioblastoma Multiforme (GBM) Brain Tumors act as complex adaptive systems or if cell-to-cell communication among tumor cells exist has not been well defined. This study aimed to assess the presence of gap junctions in primary GBM cells, and subsequently test the effect of the gap junction inhibition on tumor growth and treatment.

Using GBM cell lines and *in vitro* dye transfer assays, GBM cells were shown to communicate via gap junctions. *In vivo* subcutaneous and intracranial tumors models were used using the same GBM cell lines as used in *in vitro*. Gap junctions were found in GBM cells, and results showed that gap junction inhibition drugs reduced tumor volume, sensitized cells to chemotherapy and increased lifespans in mice that were treated with gap junction inhibitor and Temozolomide, a common chemotherapy agent compared to those that only received chemotherapy. These results show that interruption of cell-to-cell communication can potentially disrupt interactions and adaptations among tumor cells, making them more vulnerable to chemotherapy.

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Dedication

The vast majority of my research has focused on cancer treatment. This is an area of science that is actively pursued, but often slow in moving ideas or new procedures to clinics and patients. My drive to pursue a MD/PhD, and focus on moving research ‘from bench-to-bedside’ stems from my experience of family and friends who have suffered and succumbed to various cancers. It is these people who motivate me to do more experiments, study harder and ultimately achieve my career goals in the hope that, one day we will be able to provide personalized, highly effective and less toxic treatments for patients. Thus, I would like to dedicate this work, as well as all my future efforts to Opa, Tante Ruth, Sabina, Karen and all others who have been stricken with cancer.

Acknowledgments

I would like to thank my family, mentors and friends for all their help throughout this process. First of all, my mentor, Dr. Brent Reynolds, who enabled me to work in his lab for the last six years, trusting me into his lab when I knocked at his door as high school student. He has fully supported my sometimes crazy ideas and has mentored me and challenging me throughout, to become a better scientist and person. He has taught me a lot about research, life and science. Dr. Vedam-Mai has also been crucial in teaching me procedures and helping me to further understand the project. Dr. Devine has graciously been my psychology advisor and mentor and remembers me as a young science fair student many years ago. He was my faculty sponsor for my research internship last summer in Okinawa, Japan and is a great source of information and advice. I would also like to thank Dr. Ben Dunn and the Howard Hughes Medical Institute Science for Life program at UF who helped fund this research. My friends and family have been an integral part of this process as well. They have helped keep me motivated, supported me throughout and helped edit my paper. I would have not been able to accomplish all of this without everyone mentioned here. I truly appreciate each person's contributions and help with this process

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Chapter 1: Introduction

Purpose of Study

Cancer

It is estimated that approximately 23,000 new cases of brain cancer are diagnosed each year, and about 14,000 deaths annually are due to brain cancer (Cahoon, Inskip, Gridley, & Brenner, 2014). Mortality rate due to gliomas is almost 100%, with most patients living less than a year. About 50% of gliomas are Glioblastoma Multiforme (GBM) tumors, one of the most dangerous and aggressive types of tumors (Levin, Leibel, & Gutin, 2001). As the fight to cure cancer continues, more treatments have become available, and mortality rates have declined for some forms of cancer. However, brain cancer mortality has declined only by 5% in the last 50 years (Legler et al., 1999).

Despite substantial research efforts over the years, surgery and systemic therapies such as radiation and chemotherapy are still the most common treatments used. These treatments are painful, expensive, and have many side effects such as burns, hair loss, fatigue, nausea, and immunosuppression. Furthermore, systemic cancer therapies are designed to target fast-growing cells, whereas cancer stem cells (CSC) (Reya, Morrison, Clarke, & Weissman, 2001) are not affected by such treatments. Although CSCs' are not actual stem cells in the conventional sense, they mimic many stem cell properties and exhibit slow cell division rates. CSCs are resilient and are resistant to most systemic therapies that are typically designed target fast dividing cells (Vezzoni & Parmiani, 2008). The resistance of CSCs against traditional treatments has led scientists to believe that surviving CSCs are the cause of cancer relapses and metastasis (Liu et al., 2014).

Given the fact that systemic cancer treatments such as chemotherapy and radiation are generally ineffective in GBMs, and that patients receiving such treatments suffer many serious, often debilitating, side effects, managing GBM as a chronic illness (rather than trying to ‘cure cancer’) within the integrative medicine framework is becoming ever more popular (Witter & LeBas, 2008). Scientists and oncologists are also exploring the possibility of making cancer cells more susceptible to conventional treatments so as to make such treatments effective at lower doses, which can also potentially minimize the undesirable side effects. The effectiveness of both of these approaches rely on our understanding of how collection of cancer cells form primary tumors, and how tumors, which are composed of heterogeneous cells, act as single entities to cause the disease.

A recent and unorthodox thinking of a small population of cancer biologists and ecologists considers primary tumors as a complex adaptive system in which a tumor is composed of heterogeneous collection of individual cancer cells that interact with neighboring cells and adapt to their local environments; the tumor and disease it causes are viewed as emergent properties of such interactions and adaptations (Micheli-Tzanakou, 2006). Complex adaptive systems (CAS) are “fluidly changing collections of distributed interacting components that react to both their environments and to one another” (Macal & Hummel, 2014). Figure 1 shows examples of CAS in animal populations. CAS are characterized by the ability of individual components to work together to create something much larger and stronger than each individual member. For example, in an ant colony, each ant is an individual and follows a simple set of decisions

based on its job (find food, leave and/or follow a scent trail). There is no master plan or blue print for the entire colony and no one ant is in-charge of the others. Yet, the colony as a whole tends to act as a single entity, as if it were a single organism. Ant colonies are adaptable, robust, complex and quick respond to environmental perturbations; this complexity and adaptability partially explains why it is so difficult to get rid of an ant pile. Evidence suggests that collective animal behaviors such as starling murmuration and fish schooling emerge from each individual following a set of simple rules, communicating and interacting with neighbors and adapting to environmental perturbations (Cavagna et al., 2010; Hemelrijk & Hildenbrandt, 2011).

A key premise of this thinking is that cancer cells are able to interact and communicate with each other and with their micro-environments, and that disruption of cell-to-cell communication should adversely affect tumors as a complex system. However, whether or to what extent GBM act as complex adaptive systems or if cell-to-cell communication among tumor cells exist or if cell-to-cell communication is critical to structure and function of GBM tumors remains unknown. The overall goal of my research was to test two hypotheses pertaining to the existence of cell-to-cell communication in GBM brain tumor cells, and how important such communications are to conferring robustness of tumors to systemic therapies.

Scientists think that tumor cells may be communicating through gap junctions with their immediate neighbors primarily. Gap junctions are specialized channels that directly couple adjacent cells and permit the bidirectional passage of small molecules between the cytoplasm (Chipman, Mally, & Edwards, 2003).

Gap junctions

Gap junctions are made of connexins, which are in turn comprised of transmembrane domains (Figure 3). There are many different types of connexins which vary by molecular weight. These different connexins can be found in different cells throughout the body. For example, Connexin37 (Cx37), Cx40 and Cx45 are known to play a role in the heart, blood vessels and lymphatic vasculature and Cx37 and Cx40 are found in endothelial cells (Biervert & Steinlein, 1999)(Molica, Meens, Morel, & Kwak, n.d.). Some drugs tend to target one connexin more than another, and certain connexins that play an essential role in the body should not be targeted (Roell et al., 2007). The exchange of intercellular metabolites, secondary messengers, nutrients, ions, and other hydrophilic molecules under 1kDa allow neighboring cells to communicate via gap junctions and respond as a system (Chipman et al., 2003). Gap junction intercellular communication has been shown to play a role in the regulation of processes involving cell growth, tissue differentiation, maintenance of homeostasis, and embryo development (Yamasaki, Mesnil, Omori, Mironov, & Krutovskikh, 1995).

Role of gap junction in cancer

The role of gap junction coupling in Glioblastoma, and cancer in general is not well understood. Increasing recognition of the importance of gap junction communication as a major cellular function has led to the need to establish reliable and accurate methods for measuring intercellular communication (Oliveira et al., 2005). The dye transfer assay used in this study demonstrates a method for the assessment of gap junction intercellular communication by use of a modified version of the dye transfer assay developed by

Goldberg *et al* (Goldberg, J, & Naus, 1995). The advantage of the dye transfer assay demonstrated herein is that it allows for whole population analysis along with accurate quantification and high sensitivity measurements through the use of flow cytometry. Subtle effects that drugs may have on gap junction communication can be detected, whereas with visually quantified assays, e.g, microinjection, small differences in dye transfer cannot be perceived. In addition, this method allows for a minimal amount of cells to be used by adopting the assay to a 96 well plate. Thus, the possibility of screening for drugs that alter gap junction intercellular communication becomes much more practical (Lin et al., 2002). Investigations of gap junction communication within Glioblastoma have given varying results between tumor samples (Lin et al., 2002). As tumor heterogeneity is a leading concern within cancer, evaluation of the role of gap junction coupling in tumor progression, treatment resistance, and as avenues for therapy is important for furthering our understanding of tumor biology.

Statement of Hypothesis

First, I tested the hypotheses that GBM tumor cells communicate via gap junctions. Gap junctions are specialized channels that couple adjacent cells and permit bidirectional exchange of intercellular metabolites, secondary messengers, nutrients, ions, and other hydrophilic molecules under 1kDa. Although gap junction intercellular communication has been shown to play a role in the regulation of processes involving cell growth, tissue differentiation, maintenance of homeostasis, and embryo development, their roles in Glioblastoma and cancer in general is not well understood (Dere, 2012)

If GBM tumors function as complex adaptive systems, then it is reasonable to assume that cell-to-cell communication is important in structure and function of GBM tumors. Thus, I hypothesize that chemical inhibition of gap junctions should adversely affect tumors' robustness and adaptability and make cancer cells more vulnerable to chemotherapy. Specifically, I hypothesize that treatment with the gap junction inhibitor drug and chemotherapy will cause a larger reduction in tumor size than chemotherapy alone. If so, then one would expect longer lifespans in model animals treated with gap junction inhibitors (and chemotherapy) compared those treated only with chemotherapy.

Determining the existence of cell-to-cell communication in GBM brain tumor cells, was tested *in vitro* in cell cultures using primary GBM cell lines cultured in Dr. Reynolds laboratory. Once proof of concept was established and toxicity levels of each gap junction inhibitor drug are determined *in vitro*, I tested the second hypothesis, *can disruption of cell-to-cell communication in the tumor cause disruption of the tumor population, sensitizing the tumor to cancer treatment*, using *in vivo* experiments using NOD/SCID (non-obese diabetic mice with severe combined immunodeficiency disease) mice as model systems, either with a subcutaneous tumor in the right flank or intracranial tumors using the same GBM cell lines used in the aforementioned *in vitro* experiments. Test of these two hypotheses is critical in determining the existence and importance of cell-to-cell communications via gap junctions, and the possibility of improving the efficacy of systemic therapies to cure GBM cancers.

Significance of Study

If it is found that gap junctions are essential in communication and for tumor growth and resistance, it can be assumed that decreasing communication will sensitize cells. This could revolutionize the way that we study and treat tumors. Furthermore, this concept has wide application as it can be applied to most solid tumor cancers. It could potentially improve efficacy, reduce side effects and increase our capability to treat tumors and cancer. Evaluation of the role of gap junction coupling in tumor progression, treatment resistance, and as avenues for therapy is important for furthering our understanding of tumor biology. This research could also lead to advancements in personalized medicine and the ability to treat each patient based on their specific cancer with an individualized approach.

Limitations/Assumptions of study

Though the concept of gap junctions has been around for 50 years, the concept of studying gap junctions to influence cancer proliferation is new. Consequently, there are not many studies examining this hypothesis *in vitro* and *in vivo*. A large portion of the project was spent creating and optimizing protocols, determining the best way to analyze experiments and overcoming difficulties regarding drug solubility and toxicity.

Chapter 2: Methods

Passaging primary Glioblastoma Cell Lines

Passaging GBM cell lines was used in all experiments and it is important to work with healthy cells at the right cell density. Primary GBM cell lines were cultured as neurospheres following the protocol in (Deleyrolle et al., 2011). Briefly, seven days after initial plating, neurospheres are incubated in trypsin to dissociate neurospheres. Cells are then spun down, viable cells counted using trypan blue and replated or used for experiments using fresh media and EGF.

Immunostaining for Flow Cytometry

Immunostaining can be used to look at a variety of signals. In this study, immunostaining was mainly used to look at apoptosis using a pre-conjugated Caspase-3 antibody and analyzing fluorescence of the cell through flow cytometry. Immunostaining procedure is described in detail in Appendix A and in Bossy-Wetzel's paper (Bossy-wetzel & Green, 2000). Briefly, cells were dissociated from neurospheres into single cell suspension using trypsin. Cells were spun down and resuspended in 4% Paraformaldehyde (PFA) and incubated to fix cells. After 20 minutes, cells were spun down and resuspended in desired cocktail with antibodies and detergent if needed. Cells were then incubated, washed and run through the BD LSRII flow cytometer using standard protocols.

Dye Transfer Assay

To determine if GBM cells communicate via gap junctions, we developed a protocol employing flow cytometric analysis based on Goldberg's pre-loading method to evaluate gap junction communication (Goldberg et al., 1995). Cells are placed in single

suspension as described previously. Half the cell population is treated with calcein AM (a green dye), and the other half of the cell population is treated with Cell Trace Violet (a blue dye). Cells are then incubated together for 24 hours at about two million cells per milliliter to allow for direct cell-to-cell communication, and are analyzed using a BD LSRII, at various hourly intervals. Full methodology can be found in Appendix A.

Western Blot

Western blots were performed on the three GBM cell lines cultured in the lab to determine the amount of connexin 43 expression, a key indicator to evaluate presence of gap junctions using a modified protocol described by Yu (Yu et al., 2014). Briefly, GBM cells were lysed, and loaded with a protein marker mixture and loaded on the gel. The gels were blotted and membranes were transferred to a blocking buffer and agitated during incubation. Blots were washed and images were taken (Taylor, Berkelman, Yadav, & Hammond, 2013).

Temozolomide solubility and Dose Response Experiments

Temozolomide (TMZ) is the most common chemotherapy agent used to treat GBMs. It comes in a powder and is not soluble in water, so must be dissolved in DMSO. However, because DMSO is toxic, it is dissolved at the highest concentration possible (20mg/mL) and then diluted using other solvents. See Appendix A for further details.

***In vivo* experiments**

To determine the effect of gap junction inhibitors on tumor growth and survival in mice, *in vivo* experiments on mice were conducted. All animal experiments were carried out in NOD/SCID mice, under IACUC protocol number 201101502. The *in vivo*

experiments used mice (n=5): control, DMSO, TMZ, Gap Junction Inhibitor Drug A (GJIDA), and Drug B (GJIDB), and mice treated with a combination of GJIDA and TMZ. Mice were treated three times a week (Monday, Wednesday and Friday) and tumor volumes were measured five times a week (Monday through Friday) using calipers in three different axes to calculate tumor volume. Mice in the control group were treated with PBS, the mice in the TMZ group (to represent current/common therapy) were treated at 5mg/kg of ? which is the average dose used for *in vivo* experiments in laboratories. Mice in the GJIDA alone were treated with the appropriate dose as determined by the dose response experiments (delivered through the food? Drinking water?). The fourth group of mice were treated with a combination of TMZ and the GJIDA to look at possible synergistic effects. Intracranial mice were treated Monday, Wednesday, and Friday through intraperitoneal injections once the tumor reached 100mm² for the subcutaneous tumors, and two weeks after surgery for the intracranial experiments. Mice were sacrificed when tumor exceeded 1000mm², or if mouse became lethargic or ill.

Processing Tumors from mice

If the mouse had a subcutaneous tumor, the mouse was sacrificed via decapitation, and then tumor was manually removed from right flank using scalpel and tweezers. If tumor was intracranial, mouse was perfused and then brain extracted. Tumor or brain was placed in glucose or PBS until further analysis. Tumors were processed and then fixed for further downstream analysis. Details can be found in Appendix A.

Chapter 3: Results

Gap Junction Presence *in vitro*

The first goal of this project was to determine if primary GBM cells communicate via gap junctions using the dye transfer assay. Figure 4A shows the two individual cell populations at time 0 of the experiment. Half of the cells were labeled with calcein (green dye) and half were labeled with cell trace violet (blue dye). It can be seen that when these two populations were combined and immediately run through flow cytometry without incubation time, two clear populations can be visualized in the sample (Figure 4A). Over time, the cell trace violet dyed cells take up the calcein via gap junctions. As the blue dyed cells retain the green dye, a shift in the cell population can be seen as the blue cells shift right, indicating green dye within the cell as well. In 4B, it can be seen that about 20% of the cells showed dye transfer after six hours of incubation. However, when the gap junction inhibition drug was added, it decreased dye transfer by about 50%, as seen in 4C. This demonstrates the presence of drug-inhibitable gap junctions in primary GBM cells.

Toxicity levels - Dose response *in vitro*

Next, dose response curves were done to establish the toxicity of the GJID. Figure 5 shows one example of a cell count toxicity assay done to establish the dose response curve of the GJIDA. As can be seen, it follows a relatively common “S” curve on the log scale. These experiments were very important to assist in determining the doses to use in later experiments. Doses that retained 70-80% viability of cells were used for subsequent experiments.

After toxicity of the GJID were established, we wanted to discern if there were differences on the effect of the GJID on different cell lines. Three different GJID were tested on three different cell lines. It was important to use different cells lines because each GBM is different and reacts differently to the drugs. Furthermore, the GBM Cell Line S3 is TMZ resistant, making it more aggressive and difficult to treat. Figure 6 shows the data for these experiments. The data labeled GBM L0, GBM L2, and GBM S3 are the untreated controls for each respective cell line. The data shows that exposure of cells to GJID inhibited dye transfer between 25% and 90%, further proving the effectiveness of the drugs used. Asterisks indicate * $P < 0.05$.

Drug targets

Figure 7 shows that over 80% of all cells in all three cell lines expressed connexin 43, which has a molecular weight of 43-47 kDa. IHC was also performed for visual confirmation. Cell nuclei were stained with DAPI (blue) and connexin 43 (Cx43) was stained green. Figure 8 shows a very prominent presence of Cx43 in the cells, indicating that Cx43 is found widely among GBM cells.

***In vivo* experiments**

NOD/SCID mice were used for both intracranial and subcutaneous experiments. Figures 9, 10 and 11 show data from *in vivo* experiments done using the subcutaneous tumor model. In figure 9, we can clearly see the additive effective of the combination of TMZ and GJIDA. It is also interesting to note that the GJID decreased tumor growth, even without the chemotherapy drug.

To further investigate this theory, another *in vivo* intracranial experiment was performed using the GBM Line S3 cells using a similar experimental flow as previously. While the results shown in Figure 10 may not be as significant as those shown in figure 9, an additive effect was clearly shown when TMZ was used in conjunction with GJID.

***In vivo* survival curves**

Figure 11 shows a survival curve from the experiment. This provides evidence that GJID not only sensitizes GBM cells and reduces tumor volume, but it also allows increases survival of the mice. Here it can be seen that the GJID drug increased the life span about 10 days. Another gap junction inhibitor, GJI drug B, increased the survival by almost a month. Translating this to patients, the use of GJID could both reduce to rate of tumor expansion and allow the patient to live longer.

Chapter 4: Conclusion

Discussion

The data presented in this paper support the hypotheses laid out at the beginning of the paper. I tested the hypotheses that GBM tumor cells communicate via gap junctions. Through the dye transfer assays, the western blot experiment and the *in vitro* assays, it was clearly established that Glioblastoma cells communicate via connexin 43-containing gap junctions and that the process can be inhibited by specific GJIDs.

The second hypothesis tested was that if Glioblastoma cancer cells communicate via gap junctions, chemical inhibition of gap junctions should adversely affect tumors and make cancer cells more vulnerable to chemotherapy. The *in vivo* experiments clearly support the notion that chemical inhibition of gap junctions adversely affects tumor growth and progression and makes cancer cells more vulnerable to chemotherapy. This was seen through tumor volume reduction in animals treated with GJIDA and TMZ, and increased life span of mice treated with GJID.

These data provide the necessary preliminary evidence to continue to explore other gap junction inhibitor drugs with and without combination of standard chemotherapy agents. The data also provide evidence that it may be possible that cancer can modeled and treated as a complex adaptive system. Through the use of interdisciplinary research and collaborations between cancer cell biologists, physicians and ecologists, this approach could revolutionize the way we study and treat cancer.

Application

As can be seen by the minute decrease in GBM mortality in the last 50 years, there is an acute need to develop effective treatments to treat GBMs. Inhibition of gap junctions has a lot of potential to not only slow tumor growth, but to also sensitize cells to conventional treatment, allowing even the ‘resistant’ tumors to be treated more effectively. The fundamental concept regarding gap junctions and CAS is applicable to most types of cancer and has the potential to help treat and manage many types of cancer. Although the possibility of finding a “cure” for cancer is very slim, better understanding and management of the disease is the immediate goal.

If physicians focused on keeping a tumors growth at bay, and worked on improving the quality of life while managing the cancer, rather than trying to resect the whole tumor and kill every fast dividing cell in the body, including cancer cells through intense and long sessions of chemotherapy and/or radiation, the patient may be able to lead a longer, healthier and happier life.

Future Studies

As can be seen by the minute decrease in GBM mortality in the last 50 years, there is an acute need to improve the treatment of GBMs. As sequencing ability improves and cost goes down, the possibility of sequencing patients genomes and tumor biopsies provides the opportunity to personalize each patient’s treatment. Furthermore, as the complex adaptive system is better understood, there is the possibility of treating a tumor’s microenvironment (glucose levels, pH, etc) to further sensitize cells to conventional

treatments. The ability to target specific connexins related to tumor cells, and target cancer stem cells will allow for targeted and personalized treatment for cancer patients.

Tables and Figures

Tables

	Animal population	Tumor population
➤ <u>Nonlinear dynamics</u> - Small perturbation leads to large effect	Many animal populations show drastic non-linear population dynamics, but it is not always understood why	Uncontrolled proliferation and down regulated apoptosis of cancer cells
➤ <u>Self-organization</u> - Order from chaotic interactions of individuals	The organization of a bee hive or an ant pile. Murmurations and baitballs are self organized and not directed by a dominant individual or practiced as a group	Individual cells cause tumor formation and growth
➤ <u>Emergent properties</u> - Properties not obvious from properties of individuals	Each individual follows a set of simple rules, which emerges as a single behavior of the system	Individual cells may be susceptible, yet in a tumor they are robust
➤ <u>Adaptation</u> - Environment becomes encoded in rules governing structure/behavior by selection	Adaptation based on environment, predators or a change of dynamics	Tumors adapt to treatment by becoming resistant

Table 1: Characteristics shared by both animal populations and tumor populations, making it a complex adaptive system.

Figures

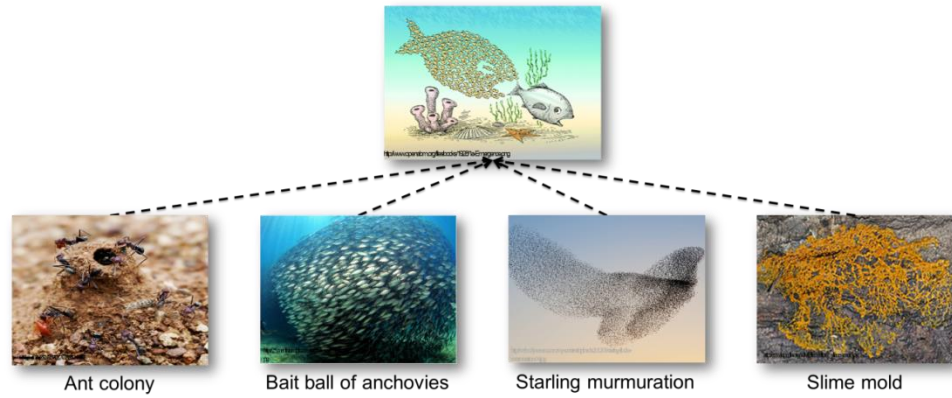


Figure 1: Examples of complex adaptive systems in nature.

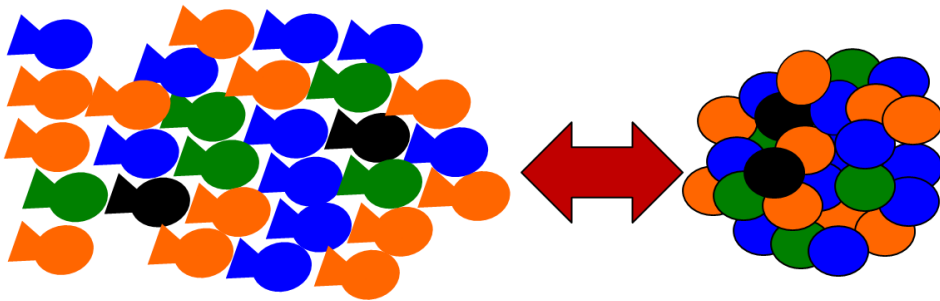


Figure 2: The relationship between a CAS in an animal population and a tumor. Here we see different types of fish (or different jobs in an ant colony) directly relates to the heterogeneity of cells within a tumor.

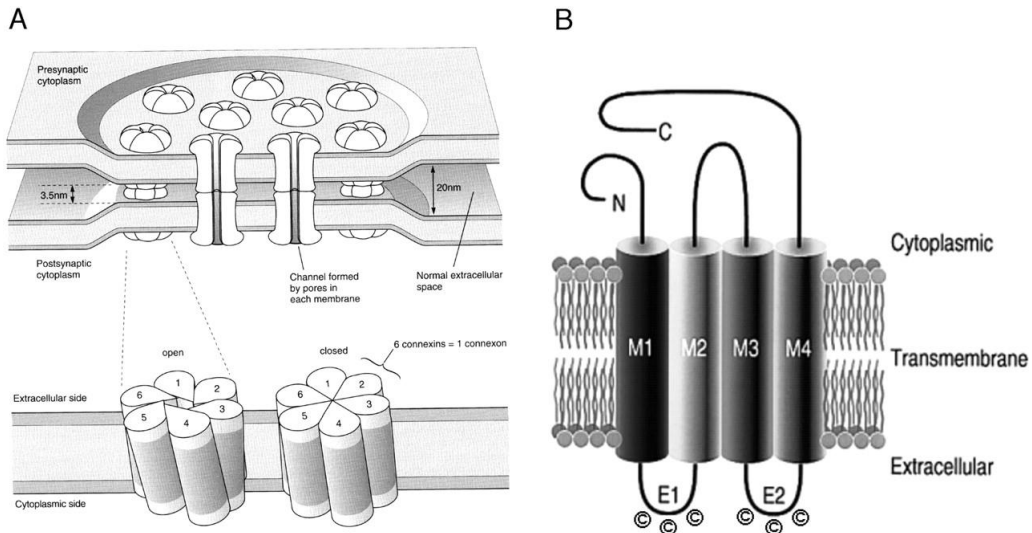


Figure 3: A) Gap junctions are made out of connexins, which are made out of (B) transmembrane domains. Notice how the gap junctions allow for direct communication between the cells (Söhl & Willecke, 2004).

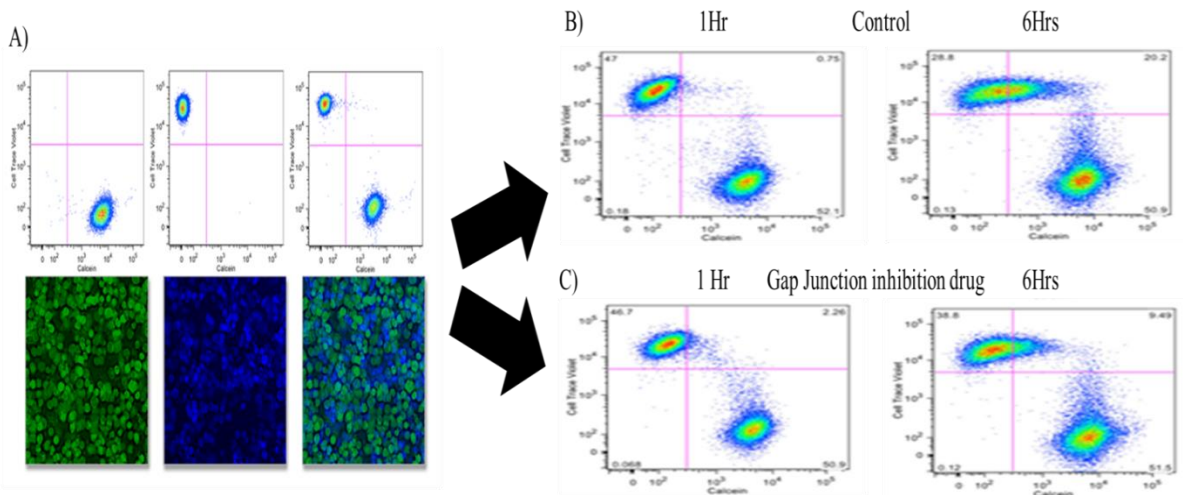


Figure 4: Proof of gap junctions in GBM cells. A) Donor cells stained with Calcein (green) dye. Acceptor cells stained with Cell Trace Violet Dye (blue). 1:1 ratio of the two populations analyzed after mixing. Assessment of dye transfer between the donor and acceptor populations over a six-hour time period. B) Control cells shows 20% dye transfer over 6 hours. C) Gap junction inhibitor drug decreased dye transfer by approximately 50%.

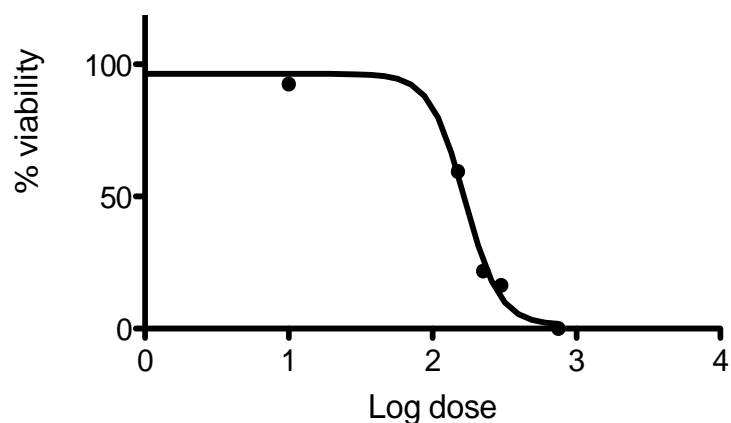


Figure 5: Dose response curves were conducted using GJIDA to understand the toxicity of the drug, and determine what concentration of the drug should be used for future studies. The x-axis shows the log dose and the y-axis shows percent viability in the cells. The dose chosen varied based on the gap junction inhibition drug used, but a dose that had about 70% viability was chosen.

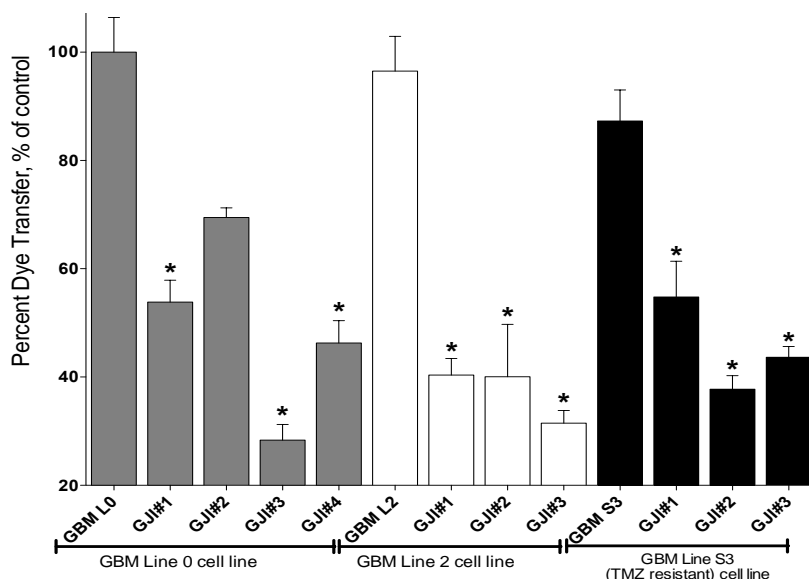


Figure 6: This shows the effect of the gap junction inhibition drugs on cell-to-cell communication. The x-axis shows the different cell lines and gap junction inhibition drugs used, and the y-axis shows the percent of dye transfer seen through flow cytometry, as compared to the control. Once toxicity and ideal dose were determined, the effectiveness of the GJID on three cell lines were tested to examine the effectiveness of different drugs on various types of cells. The asterisks indicate a p-value of $*P < 0.05$ as compared to control cells.

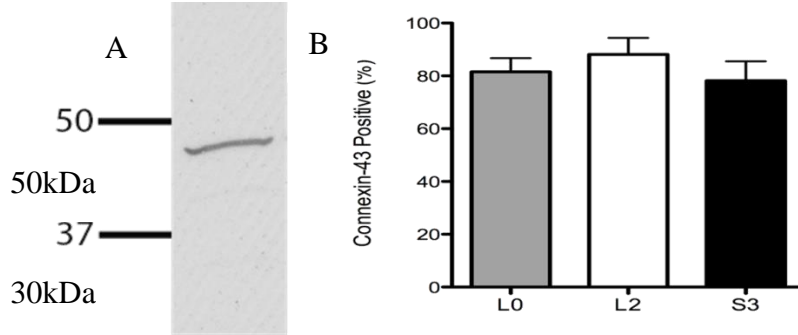


Figure 7: (A) This is a representative gel which demonstrates the size of the connexin (~43kDa) on a Western blot. (B) Western blot analysis of Cx43 in GBM. Cx43 antibody and secondary anti-mouse horseradish peroxidase antibody were used. Experiment was performed on three cell lines. As can be seen, over 80% of all the cells expressed connexin 43.

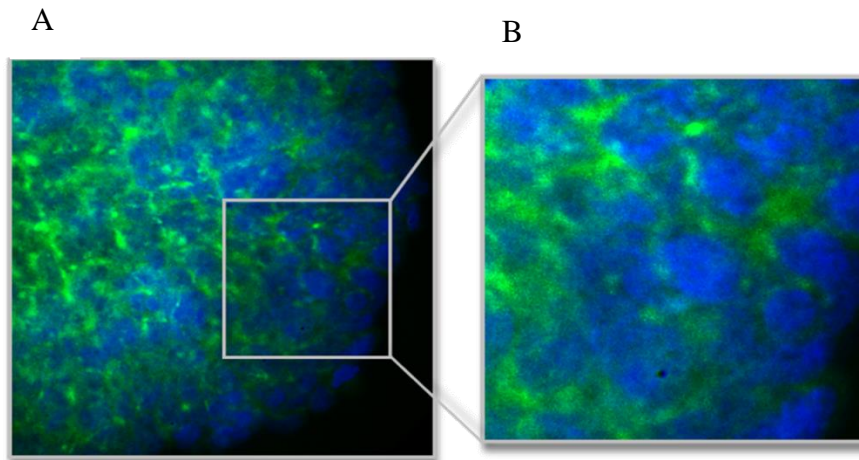


Figure 8: A representative GBM neurosphere stained for connexin-43 (green) and cell nuclei stained with DAPI (blue). (B) shows a magnified view of (A). As can be visually seen, the majority of the cells seem to express connexin-43.

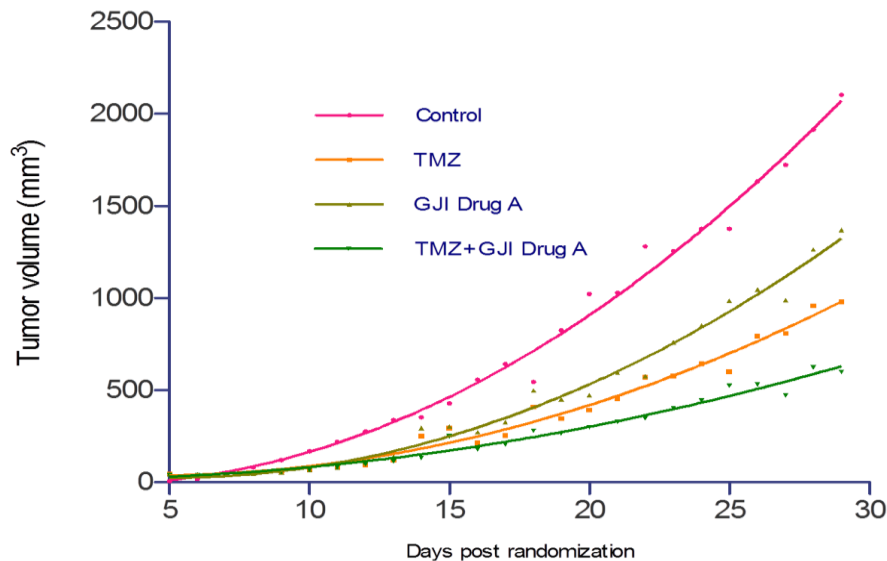


Figure 9: In vivo data showing tumor volume over time. TMZ is the most common chemotherapy drug used to treat GBMs. The x-axis shows days post randomization, that is also defined as two weeks after implanting the GBM cells into the tumor, and also when tumor volume measurements began. The y-axis is the average tumor volume in mm^3 for that group of mice. GJI Drug A is effective at tumor knockdown and TMZ+GJI Drug A had an additive effect on tumor volume as compared to the control.

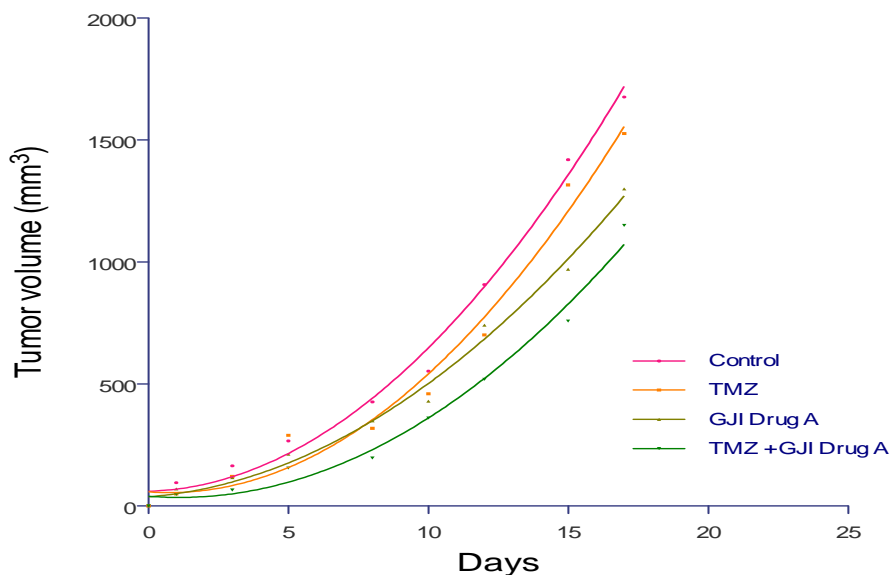


Figure 10: This shows the tumor volume in mice that were transplanted with the S3 cell line, which is resistant to chemotherapy. The x-axis shows days post randomization, that is also defined as two weeks after implanting the GBM cells into the tumor, and also when tumor volume measurements began. The y-axis is the average tumor volume in mm^3 for that group of mice. GJI Drug A shown to have an effect on TMZ resistant cell. In combination with TMZ, GJI Drug A was capable of sensitizing the resistant cells to TMZ.

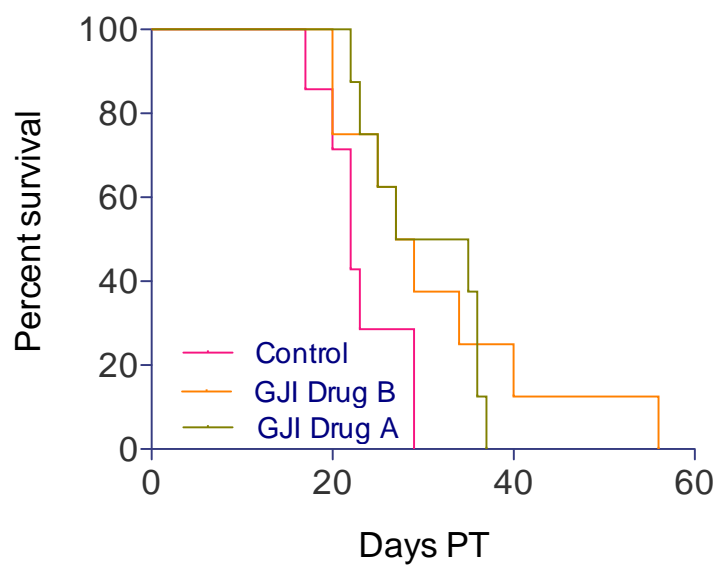


Figure 11: In a more clinically relevant model, GJI Drugs A and B extend the life of mice implanted with brain tumors intracranially. The x-axis shows days post transplant of the GBM cells. The y-axis is the percent of the group (n=5) who were still alive. As can be seen, the gap junction inhibition drug A and B increased the life of the mice by about ten to thirty days.

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Appendix A: Acronyms

- ABX - Antibiotics
- BD LSRII - A flow cytometer equipped with 4 lasers
- Calcein AM - A cell-permeant dye that can be used to determine cell viability
- CAS - Complex adaptive systems
- Casp3 – Caspase 3 - Apoptotic cell marker
- CO₂ – Carbon dioxide
- CSC - Cancer stem cells
- DMSO - Dimethyl sulfoxide
- EDTA - Ethylenediaminetetraacetic acid
- EGF – Epidermal Growth factor
- ETOH – Ethanol
- FBS - Fetal bovine serum
- FSC– Forward side scatter light in a flow cytometer
- GBM - Glioblastoma multiforme
- GJID - Gap junction inhibition drugs

- IHC –Immunohistochemistry
- KDa - Kilo Dalton
- KI67 – Proliferation cell marker
- L0 – A primary Glioblastoma multiforme cell line
- L2 – A primary Glioblastoma multiforme cell line
- MBI – Cell culture media
- MCT – Medium chain triglycerides oil
- NGS – Normal goat serum
- NOD/SCID Mice- Non-obese diabetic mice with severe combined immunodeficiency disease
- NSA- Serum-free basal medium for the culture of human neural stem cells
- PBS – Phosphate buffered saline
- PE – Phycoerythrin, florescent dye
- PFA – Paraformaldehyde
- RPM – Rotation per minute
- S3 – A primary Glioblastoma multiforme cell line, TMZ resistant
- SSC- Side scattered light in a flow cytometer
- T - Trypsin
- TI - Trypsin Inhibitor
- TMZ – Temozolomide, an oral chemotherapy drug
- x g - Centrifugal force, centrifuge speed

Appendix B: Detailed Methods

Passaging primary Glioblastoma Cell Lines

Primary GBM cell lines were cultured as neurospheres following the a standard protocol (Deleyrolle et al., 2011). Briefly, seven days after initial plating, neurospheres are incubated in trypsin to dissociate neurospheres. Cells are then spun down, counted and replated using fresh media and EGF.

GBM Cell lines

L0 - A primary GBM cell line. It reaches confluency after about seven days, and is not known to be resistant to TMZ.

L2 - A primary GBM cell line. It reaches confluency after about seven days, and is not known to be resistant to TMZ.

S3 - A primary GBM cell line. It reaches confluency after about five days, and is resistant to TMZ.

GBM Treatments

TMZ-Temozolomide is a common chemotherapy agent used to treat patients with GBMs
GJI Drug A- Gap Junction Inhibitor Drug A is a name that was assigned to one of the drugs we used as a gap junction inhibitor. Due to proprietary reasons we are unable to use the actual name of the drug.

GJI Drug B- Gap Junction Inhibitor Drug B is a name that was assigned to one of the drugs we used as a gap junction inhibitor. Due to proprietary reasons we are unable to use the actual name of the drug.

Immunohistochemistry staining of neurospheres

DAPI (blue)

Annexin-43 (green)

Dye Transfer Assay and Immunostaining for Fluorescent Activated Cell Sorting

1. Prepare Cells from incubator.
 - a. Put trypsin and trypsin inhibitor in water bath to warm up.
 - b. Work with cells only in tissue culture hood
 - c. Take flask with neurosphere cells (GBML0, treated with 30 μ L 2mg/mL TMZ and 20 μ L DMSO) out of the incubator.
 - d. Label tube. Transfer cells into 15ml tube. Spin at 500rpm for 5min. Aspirate using glass pipette.
 - e. Resuspend in 1mL trypsin (0.25% trypsin) pipette up and down to mix well. Place in water bath at 37⁰C for 3min
 - f. Add 1mL trypsin inhibitor. Pipette very well. Should be single cell. If not, spin and repeat trypsin step.
 - g. Spin at room temperature at 500rpm at 5min. Aspirate using glass pipette.
 - h. Take out of hood. Resuspend thoroughly in 1mL 4% PFA.

- i. Transfer to 1.5mL Eppendorf tube. Incubate at room temperature for 20min.
 - j. Label tube. Spin at 1200rpm for 5min. Aspirate using glass pipette. Resuspend in 1mL PBS.
 - k. Do cell count: Take a new Eppendorf tube. Add 90 μ L trypan blue (0.4%), and 10 μ L cells (mix by pipetting up and down). Inject 10 μ L into hemocytometer. Count. If 1 quadrant has less than 50 cells, count all four quadrants and divide that number by 4, then multiply by 10^5 . (ex: if cell count for 4 quadrants is 107, then calculation is $(107/4) \times (1 \times 10^5) = 2.675 \times 10^6$ (~2.6 million cells) and you will have the number of cells in your 1mL of PFA.
 - l. Do a cell count for each cell line separately.
2. Staining of Cell lines
 - a. Compare cell lines: L0 negative control and treated L0.
 - b. Label tube. Prepare 1 million cells in 1mL: Take x μ L L from L0 and transfer to new tube. Resuspend in PBS to fill up to 1 ml. Split each cell line into 2 samples.
 - c. Using 15ml tube, add 2mL NGS/PBS/Triton for each cell line. Label tube.
 - d. Retrieve Casp3 and KI67 from antibody fridge in box. Make sure to keep in dark. Casp3 and KI67 are used at 1:1000.
 - e. Add 2 μ L of casp3 and Ki67 to NGS/PBS/Triton. MIX WELL. Keep in dark
 3. Stain samples
 - a. Take 0.5 ml cell samples from before.
 - b. Spin at 1200rpm 5min.
 - c. Aspirate using glass pipette
 - d. Resuspend one of each cell line in 1000 μ L NGS/PBS/TritonX/Casp3/KI67. For “unstained control” resuspend one of each cell line in NGS/PBS/Triton ONLY! NO ANTIBODIES. Label tubes carefully label one “stained treated” and one “unstained treated” and one “stained untreated” and “unstained untreated”.
 - e. Incubate at RT or 37C for 1hr in the dark.
 - f. While incubating, label 4 FLOW CYTOMETRY tubes label one “stained treated” and one “unstained treated” and one “stained untreated” and “unstained untreated”.
 - g. Turn on FLOW CYTOMETRY machine. If flow computer is empty, prepare new experiment and label each sample on the computer beforehand.
 - h. Spin at 1200rpm for 5 min. Aspirate using pipette. Resuspend in 500 μ L PBS and transfer directly into correlating FLOW CYTOMETRY tube.
 4. Run FLOW CYTOMETRY
 - a. Set up experiment.
 - b. Try to collect as many data points as possible (> 10,000 cells per sample)
 - c. Run unstained untreated cells first as baseline. Adjust FSC/SSC/FITC/PE
 - d. Run stained untreated cells. Use separate protocol to collect FLOW CYTOMETRY samples.

- e. Run stained treated cells and unstained treated cells DO NOT CHANGE SETTINGS.

Dye Transfer Assay

The assay can be performed in various different size cluster well plates and conducted through a variety of time courses. A 96-well plate format is demonstrated in this procedure to illustrate the use of a low number of cells. Six 1-hour intervals are evaluated in conjunction with a negative control gap junction inhibited series. Any variation in assay scale should maintain the same ratios.

1. Dissociation of Gliomaspheres into Single Cell Suspension
 - a. Human gliosphere culture is maintained using the neurosphere assay. At the appropriate time for passaging the gliomaspheres, collect the culture and transfer it to a sterile tissue culture tube.
 - b. Centrifuge cells at 110 x g for 5 minutes.
 - c. After discarding the supernatant, resuspend the gliosphere pellet in 1 mL of 0.05% trypsin-EDTA and incubate in a 37 °C water bath for 2-5 min.
 - d. Add 1 mL of trypsin inhibitor and gently pipette up and down to ensure homogeneity and complete neutralization of trypsin activity.
 - e. Centrifuge cells at 110 x g for 5 minutes.
 - f. Discard the supernatant then resuspend the cell pellet in 1-2 mL of NeuroCult® NSA Basal Medium.
 - g. Perform a cell count by mixing 10µl of the single-cell suspension with 90µl of trypan blue.
2. Staining Donor and Acceptor Cell Populations
 - a. Transfer an equal number of cells from the single cell suspension to two tissue culture tubes for staining of the donor and acceptor cell populations.
 - b. Prepare the donor and acceptor staining reagents in a volume that will be added to the donor and acceptor cell suspensions to give a concentration of 10⁶ cells/mL. The donor staining solution is prepared by diluting 1µL of 2mM Calcein AM dye stock solution per 1 mL of NeuroCult® NSA Basal Medium. The acceptor staining reagent is prepared by diluting 1µL of 5mM Cell Trace™ Violet dye stock solution per 1 mL of NeuroCult® NSA Basal Medium.
 - c. Add the donor staining reagent to the tube containing the donor cell suspension to give a final working concentration of 2 µM Calcein AM. Then, add the acceptor staining reagent to the tube containing the acceptor cell suspension to give a final working concentration of 5 µM Cell Trace™ Violet dye. Both donor and acceptor cell solutions should now be at a concentration of 10⁶ cells/mL
 - d. Incubate both cell solutions for 8 minutes in a 37 °C water bath, protected from light.

- e. Quench both staining solutions by adding 4-5 times the original staining volume of NeuroCult® NSA Basal Medium and then centrifuge at 110 x g for 5 min.
 - f. Discard the supernatant, then resuspend the cell pellets in 5 mL of NeuroCult® NSA Basal Medium with NeuroCult® NSA Proliferation Supplement. Incubate in a 37 °C water bath for 30 minutes to allow any excess dye to purge from cells.
 - g. Next, centrifuge the cells at 110 x g for 5 minutes, and subsequently discard the supernatant.
 - h. Resuspend both the donor and acceptor populations in 0.5-1 mL of NeuroCult® NSA Basal Medium containing NeuroCult® NSA Proliferation supplement, 1% fetal bovine serum (FBS), and 20 ng/mL epidermal growth factor (EGF).
 - i. Perform a cell count for each cell suspensions by diluting 10 µL cells in 90 µL of trypan blue.
3. Combining Donor and Acceptor Cell Populations to Assess Gap Junction Mediated Dye Transfer
- a. Combine 1.2×10^6 cells from the donor population and 1.2×10^6 cells from the acceptor population to a new tissue culture tube, i.e. 1×10^5 cells from the donor population and 1×10^5 cells from the acceptor population for every well required in the 96-well plate.
 - b. Add enough prepared NeuroCult® NSA Basal Medium containing proliferation supplement, 1% FBS, and 20 ng/ml EGF so that the final volume contains 2.0×10^6 cells/ml.
 - c. Add 100 µL of the combined donor and acceptor cell solution into six wells of a 96-well plate, i.e. one well for every 1-hour time interval. Cells should be at a density of approximately 3.1×10^4 cells/mm² well surface area.
 - d. Add enough of a suitable gap junction inhibitor to the remaining combined donor and acceptor cell solution in order to bring the inhibitor to its working concentration.
 - e. Add 100 µL of the combined donor and acceptor cell solution with gap junction inhibitor into six wells of the 96-well plate.
 - f. Place the cluster-well plate in a 37 °C, 5% CO₂ humidified incubator and begin recording the time.
 - g. At the necessary time intervals, add 100 µL of 0.05% trypsin-EDTA to the appropriate wells and incubate at 37 °C for 2-5 minutes.
 - h. Add 100 µL of soybean trypsin inhibitor and then harvest cells into a micro-centrifuge tube. Centrifuge cells at 110 x g for 5 minutes and subsequently discard the supernatant.
 - i. Resuspend cells in 0.5 mL of a 4% formalin solution and allow cells to fix for 15 minutes before centrifuging at 185 x g for 5 minutes.
 - j. Finally, wash cells with 1 mL phosphate buffered saline (PBS), and store in PBS protected from light until ready for evaluation by flow cytometry.

4. Detection of Gap Junction Intercellular Communication by Flow Cytometry
 - a. Make sure suitable fluorescent detectors are activated on the flow cytometer to record the emission spectra for both Calcein and Cell Trace Violet dye. In this procedure, the 530/30 and 450/50 nm bandwidth detectors on a BD LSRII flow cytometer are used, shown in the video as Pacific Blue and CFSE detectors. In addition, activate Forward Scatter and Side Scatter – Height and Width values to be recorded.
 - b. Construct a plot of side scatter area (SSC-A) versus forward scatter area (FSC-A), then run a non-stained sample of cells through the flow cytometer. Adjust SSC-A and FSC-A voltage settings as needed to center the cell population in the plot.
 - c. Construct a plot of Cell Trace Violet dye versus calcein and run one of the 1:1 ratio samples through the flow cytometer. Adjust the voltage settings accordingly so that both the non-stained cell population and stained donor and acceptor populations are visible within the plot. Bi-exponential axes may need to be applied to the graph.
 - d. Record flow cytometry events for non-stained control cells, for the six hour time interval series of donor and acceptor populations, and for negative control gap junction inhibited time trials.
 - e. Analyze flow cytometry data by gating the main cell population. Next, select for singlet cell events within the main cell population by constructing a plot of Side Scatter Height (SSC-H) versus Side Scatter Width (SSC-W) and gating the singlet event population, see video. Analyze the selected population further by constructing a plot of Forward Scatter Height (FSC-H) versus Forward Scatter Width (FSC-W) and gating for the singlet event population, see video.
 - f. Based on the first 1-hour time interval, construct a quadrant gate that excludes the donor and acceptor populations from the top right quadrant, as shown in Figure 3-B. Apply this gate to all other samples and controls throughout the six hour time period. Using the 1-hour time interval as the starting point for dye transfer allows for any initial dye present to be taken into account.
 - g. Calculate the percent dye transfer to the acceptor population by dividing the number of acceptor cells obtaining calcein dye by the overall number of cells in the acceptor population.

Temozolomide

Caution: TMZ should be treated as a carcinogen – use appropriate caution.

Reconstitution should be done in the hood. The half life of TMZ is very short (less than an hour) so you must work quickly for this.

1. Carefully open the TMZ bottle. Try to make sure the powder is not stuck to the top cover.
2. Pipette 5mL of DMSO into the TMZ bottle. DO NOT PIPETTE or stick the pipette tip all the way into the TMZ bottle.

3. Sterilize a stir bar (using bleach/ethanol/DI water) and place into the TMZ bottle.
4. Replace cap of TMZ bottle
5. Place on spin plate for ~10min.
6. Ensure that the TMZ is completely dissolved.
7. Dilute (in medium, DMSO, MCT, etc) and aliquot. Label aliquots carefully.
8. Freeze in the -20°C as soon as possible.

In vivo experiments

All animal experiments were carried out in **Non-obese diabetic mice with severe combined immunodeficiency disease** (NOD/SCID mice), approved by IACUC protocol number 201101502. The *in vivo* experiments used various treatment groups (n=5): controls, DMSO, GJID, TMZ+GJID. Mice were treated Monday, Wednesday, and Friday through intraperitoneal injections once the tumor reached 100mm^2 for the subcutaneous tumors, and two weeks after surgery for the intracranial/intracranial experiments. Mice were sacrificed when tumor exceeds 1000mm^2 , or if mouse became lethargic or ill. Tumors were processed and then fixed for further analysis using above methods.

Excising and Processing Tumors from mice

Excising tumors

1. Kill mouse by appropriate method
2. One animal is sacrificed, it is extremely important to move quickly and efficiently.
3. Extract tumor using tweezers, scissors and scalpel. Spray down area around tumor with ethanol, ensuring the fur is wet. Make an incision rostral to the tumor. Cut the skin around the tumor until the tumor is fully exposed. Carefully using the scalpel or scissors, beginning cutting the connective tissue and filaments between the tumor and skin, and tumor and muscle (it is easier to separate tumor from skin first). Once the tumor is completely cut away, make sure there are no hair on it. Cut the tumor in half. Take one half of the tumor and cut in half again, put 2 x $\frac{1}{4}$ pieces into labeled cryotubes. Take the other half of the tumor and place in a 50ml conical tube with 1ml PBS
4. Snap freeze tumor. Fill up a durable container with liquid nitrogen. Place cryotubes in liquid nitrogen for ~30-45 seconds. Remove cryotubes from liquid nitrogen with long tweezers and immediately place in -80°C freezer.
5. If processing more than 1 tumor, place cryotubes on dry ice until all tumors are processed, then transfer all samples to 80°C freezer at once. Label each tube carefully.

Tumor processing

1. Process tumor into single cell suspension. Take a 50mL conical tube, put 2mL trypsin inhibitor in it. Put a 20 micron filter on the top. Put on ice. Place the petri dish on ice. Place tumor in petri dish (no PBS). Using scalpels, chop up tumor into pulp. After 5min of vigorous chopping, add 0.5mL trypsin. Keep chopping. Once in a fine pulp, cut the tip off of a 1mL pipette and transfer the tumor pulp to the filter. Agitate the tumor pulp and push through filter, adding ~1mL trypsin if necessary. If tumor is still

- in large chunks/won't go through filter, transfer back to petri dish, add more trypsin and keep cutting.
2. Transfer trypsin, trypsin inhibitor and cells into 15mL conical tube.
 3. Spin at 1200rpm 5min. Remove supernatant and transfer into new 15ml conical.
 4. Spin at 1200rpm 5min. While spinning, add 1mL PFA to cell pellet and resuspend pellet.
 5. Remove supernatant and add 1mL 4% PFA to second tube. Transfer the new pellet in PFA to the original tube (already containing the original cell pellet and PFA). There should now be single cells in 2mL of 4% PFA.
 6. Place on rocker and incubate at room temperature for 30min.
 7. Spin at 1200rpm 5min
 8. Remove supernatant and resuspend 1mL PBS. Use cells for a variety of analyses.