Behavior of Glioblastoma Cells in Co Culture with Rat Astrocytes on an Electrospun Fiber Scaffold

THESIS

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Abstract

Gliomas are the most common brain tumor in adults, occurring in more than 50% of brain tumor cases. Astrocytomas, or tumors that arise from the astrocytes of the brain, account for 60-70% glioma cases.[1] The most common form of glioma is the grade IV astrocytoma known as glioblastoma multiforme.[2] The interactions between glioblastoma cells and normal astrocytes have not been thoroughly explored in a three dimensional environment *in vitro*. This research investigated the co culture of human glioblastoma cells and rat astrocytes in different ratios on electrospun polycaprolactone (PCL) fibers as well as the culture of human glioblastoma cells and rat astrocytes individually on PCL fibers. In particular, the migration of glioblastma cells and GFAP expression of rat astrocytes were measured in both co culture conditions and individual cultures of the cell of interest. The reactivity of astrocytes and the migration of glioblastoma cells was also investigated in the presence of individual factors thought to be affecting behavior of these cells in co culture. The results were then compared in an attempt to identify cell behavioral differences between different conditions. Glioblastoma migration was increased when cells were co cultured with astrocytes and cultured in media conditioned with rat astrocyte released soluble factors. GFAP expression was upregulated in rat astrocytes cultured with glioblastoma cells versus rat astrocytes cultured alone.

Dedication

This document is dedicated to my family, friends, and fiance.

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Chapter 1: Introduction

The glioma is a highly dangerous form of brain tumor found in more than 50% of brain tumor cases in adults.[1] Even with surgical removal of the glioma tumor and post-surgery radiation therapy, the mean survival time of patients is only 12 months.[3] Astrocytomas account for approximately 60-70% of gliomas, and the most common of these is the grade IV astrocytoma known as glioblastoma multiforme (GBM)(Figure 1).[1,3]

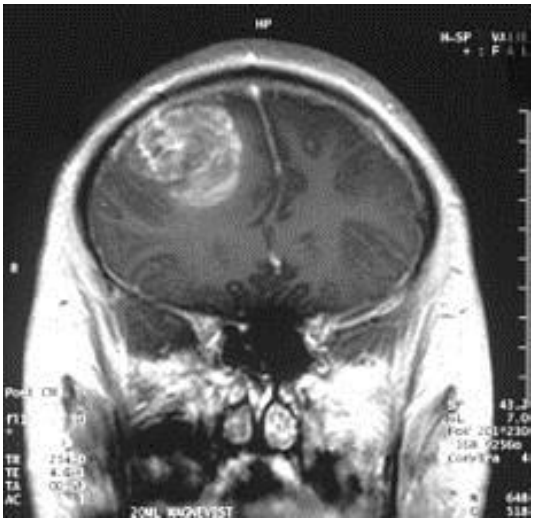

Figure 1: Magnetic Resonance Imaging (MRI) of Glioblastoma Multiforme (image taken directly from Wikipedia.org)[4]

Current GBM treatment calls for surgical resection of the tumor followed by a combination of radiation and chemotherapy for up to 6 months after surgery.[5] Even with use of these treatments, GBMs remain incurable[6] and the mean survival time of

patients diagnosed with GBMs is only 8-16 months.[7] There are several issues that prevent highly successful treatment using these therapies. One issue is that GBM cells develop alterations that make them highly resistant to the cell death pathways induced by radiation and chemotherapy.[8] Another major issue is the fact that chemotherapy reagents have difficulty crossing the blood brain barrier and penetrating the brain tissue.[9] Another issue is that one cannot keep on removing pieces of the brain, eliminating the possibility of repetitive surgical removals of tumors. The major issue that this work investigated is that GBMs show high levels of invasive behavior. Individual GBM cells have been shown to possess the ability to migrate away from the primary tumor mass into surrounding areas of the brain. These invading and migrating tumor cells typically travel along the brain's white matter tracks and along the exterior of blood vessels[1] and cause growth of secondary tumors after surgical removal of the primary tumor.[10] Additionally, there is evidence that using radiation treatment actually enhances GBM ability to invade and migrate.[11] Clearly there is a need to gain a better understanding of GBM migration in the brain to develop more effective ways to combat this migration and thus GBMs in general.

There are several methods currently used to investigate migration and invasion of GBM cells *in vitro*. These methods include the monolayer wound healing assay, the microliter-scale migration assay, the transwell migration assay, and the brain slice invasion assay.[12] A monolayer wound healing assay is performed on a two-dimensional tissue culture dish. A monolayer of cells is grown to confluence on the dish, and a

scratch wound of about 400 μm width is made in the monolayer. Images of the wound are then taken over a 12-24 hour period and analyzed to determine migration distance of cells at the wound edge.[12] The micro liter-scale migration assay is used to measure migration in response to the presence of certain ECM components. It is performed by coating Teflon-printed microscope slides with the desired ECM component, seeding cells on the slides, and measuring cell migration using an inverted microscope.[12] Another method to measure migration of cells is by use of a transwell migration assay. It consists of a bottom chamber containing media or some other substance to attract cells and a small insert in the bottom chamber containing a porous membrane of variable pore size with the cells in question seeded on top of the membrane. The cells then migrate through the membrane to the lower chamber and the number of migratory cells is counted.[13] Finally, the brain slice invasion assay is also a common method for investigating GBM migration. This method is performed by removing rat brains and cutting the cerebrum into 400 μm thick slices. Cells stained with fluorescent dye are then seeded onto the brain slices and lateral migration of cells on top of the brain slice can then be tracked using immunofluorescent microscopy.[12] Whereas these are all valuable methods to study GBM migration, they each have their shortcomings. They all examine GBM migration in a two-dimensional (2D) environment which does not closely mimic that utilized by GBMs to migrate *in vivo*. Whereas the brain slice method uses part of an environment that would be encountered by GBM cells *in vivo*, it is hard to get reproducible results using this method and has variables that are difficult to control,

such as degradation of myelin, which can influence results.[14] Additionally, brain slices differ greatly between species and also between different subjects of the same species. Thus, it is clear that there is a need to develop 3D migration systems that more closely mimic the *in vivo* environment experienced by GBMs and those that reproduce the phenotypes and genotypes typically evidenced *in vivo*.

As mentioned previously, it has been observed that GBM cells migrate along the white matter tracks and blood vessel periphery in the brain. To mimic this native environment we used electrospun poly (caprolactone) (PCL) fiber scaffolds in an attempt to create a more physiologically relevant three dimensional system to study GBM migration. The fibers used in this work were meant to mimic the topography and mechanics of the white matter tracts seen in the brain. White matter tracts consist of fibers with average diameters of 0.5-3 μm and average densities of 10000-30000 fibers/mm^2 *in vivo*. [1516] By comparison, our PCL fibers have an average diameter of 1.17±0.26 μm and an average density of 24938±5312 fibers/mm^2. Additionally, the nerve fibers that compose white matter tracts in the peripheral nervous system (PNS) have a stiffness of 0.1-1.5 MPa as reported by AFM,[17] whereas our PCL fibers have a stiffness of approximately 8 MPa. Furthermore, because GBMs arise from the normal astrocytes of the brain, the interactions between these two cells are of great interest. While previous work has studied such interactions in 2D systems[12 18], there are very few studies that have examined these interactions in 3D systems. Thus, co culture of normal and cancer cells in 3D biomimetic models is an understudied area in cancer biology. To

continue to fill this gap and better understand dynamic behaviors of tumor cells co cultured with normal cells, this research investigated these interactions (i.e. GBM migration as well as astrocyte behaviors) under biomimetic *in vitro* microenvironments. Additionally, most studies of this nature have used GBM cells derived from cell lines that have been maintained for extensive periods of time and thus may have mutated, causing them to behave much differently than they would have *in vivo*. In this work we used a GBM cell line obtained from a patient at The Ohio State University Medical Center that is less like to be mutated from its *in vivo* form than cell lines used in other work.

An astrocyte that is reacting to a CNS trauma is called a reactive astrocyte and the overall astrocytic response to this trauma is termed reactive astrogliosis. The major indicator of an astrocyte undergoing reactive astrogliosis is a substantial increase in synthesis and expression glial fibrillary acidic protein (GFAP), which is the major intermediate filament found in astrocytes.[19] GFAP is also upregulated in astrocytes surrounding invading brain tumors *in vivo*.[20] Additionally, previous astrocyte-GBM co culture studies *in vitro*, albeit using different systems than the one used in this research, also found an up regulation of GFAP in astrocytes in presence of GBM cells.[18] Because of GFAP's role as an astrocytic response to tumors of the CNS and previous evidence of GFAP up regulation in co culture systems, we initially investigated GFAP expression as an indicator of interaction between GBM cells and astrocytes in our co culture system.

Next we investigated the influence of the presence of normal astrocytes on GBM migration behaviors. GBMs were co cultured with rat astrocytes in conditions of different rat astrocyte to GBM ratios to study the effects of the physical presence of astrocytes on GBM migration. GBMs were also cultured in rat astrocyte conditioned media to investigate the effects of astrocyte-released soluble factors on GBM migration. Both the co culture and astrocyte conditioned media migration responses were compared to the response in GBM-only culture in unconditioned media (control). In addition to utilizing the previously mentioned white matter tracks and blood vessel periphery, it is also known that GBMs interact with the surrounding normal brain extracellular matrix (ECM)[21] and synthesize proteins to establish their own extracellular matrix as a means to assist in migration.[22] Studies have shown that as the rigidity and density of the ECM increases, GBM migration speed also increases.[23] Studies have also shown that addition of certain ECM components, including laminin, fibronectin, collagen type IV, and vitronectin, to culture medium stimulates migration of glioma cells.[24] To investigate the interactions between GBMs and surrounding normal brain ECM, we cultured rat astrocytes on fiber scaffolds to allow them to secrete proteins and deposit ECM onto the fibers. Astrocyte cells were then removed from the fibers while retaining deposited ECM. GBMs were then seeded onto the ECM-fiber scaffold and migration rates were measured and compared with those from fibers not containing astrocyte deposited ECM.

Chapter 2 provides details regarding the development and functions of astrocytes, the differences between GBM cells and astrocytes, the composition of the brain ECM, the molecular signaling pathways responsible for GBM migration, and prior research into glioma and astrocyte behaviors in co culture systems that were used as inspiration for this research.

Chapter 3 describes in detail the experimental protocols and results of this research. Astrocyte expression of GFAP increased with an increasing presence of GBM cells. Interestingly both the presence of astrocytes and the presence of astrocyte released soluble factors induced increased migration of GBM cells, whereas the physical presence of astrocytes caused GBM migration to be reduced.

Chapter 4 discusses the important implications of this work, particularly the factors affecting GFAP expression of rat astrocytes and migration of GBM cells, and identifies areas for future research.

Chapter 2: Background

To investigate GBM-astrocyte interactions, it is important to understand these cell types and their associated functions. Because GBM cells arise from astrocytes, understanding the differences between GBMs and astrocytes as well as the differences between GBMs and other grades of astrocytomas is crucial. This chapter will discuss astrocytes, their functions, and differences from their tumor counterparts. Next, the composition of the ECM of the normal brain and also the composition of ECM in the cancerous brain will be presented. Additionally, the physical mechanisms of GBM migration as well as the molecular signaling pathways that have been shown to play a major role in GBM migration will be highlighted. Finally, other studies involving co culture of GBMs and astrocytes and their findings will be discussed.

2.1: Astrocytes

Astrocytes outnumber any other cell type in the central nervous system (CNS).[25] Astrocytes, along with oligodendrocytes, are one of the two main macroglial cells in the CNS.[26] Astrocytes first arise as neural stem cells, or neural precursor cells, located in the subventricular zone (SVZ) of the developing brain.[27] The SVZ is a region of dividing cells along the lateral wall of the lateral ventricles that houses several CNS cell types, including astrocytes and neuroblasts in addition to neural precursor cells, and is the location for growth of new neurons destined for the olfactory bulb.[28] Certain precursor cells migrate away from the SVZ and differentiate into astrocytes after exiting the SVZ

and being exposed to factors released locally in grey and white matter in the brain which induce astrocyte differentiation.[25] These factors that induce differentiation of neural precursor cells into astrocytes include bone morphogenetic proteins (BMPs), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF).[27] Astrocyte differentiation is induced by these factors via the Janus kinase signal (JNK) transducer and via activation of the transcription 3 pathway.[29] The level of differentiation of neural precursors into astrocytes can be identified by looking at several factors and major intermediate filaments being expressed by the astrocyte. The earliest markers of differentiation from neural precursor cell to glial precursor cell are A2B5 and 4D4.[30] Studies have indicated that the earliest markers of glial precursor cells differentiating into astrocyte precursor cells are CD44, nestin, S-100β, and GFAP, whose expression is initially low and increases as differentiation progresses towards fully mature astrocytes.[30] In astrocyte precursor cells, the major intermediate filament that is expressed in vimentin, but as precursor cells approach becoming fully differentiated astrocytes there is a switch to GFAP as the major intermediate filament being expressed, which is a major indicator of astrocyte maturity.[31,32]

In terms of morphology *in vivo*, mature astrocytes are highly heterogeneous. For example, it has been shown that up to 9 different astrocyte morphologies can exist in one brain region.[33] In general, however, astrocytes in the normal, uninjured brain take on a star-like shape with long, thin processes extending out from the cell body.

Astrocytes can change their morphology in certain situations (e.g. CNS trauma) and these morphological changes can affect neuronal synaptic activity.[34]

Over the past several decades, astrocytes have been discovered to have many important functions in the CNS. Astrocytes were initially thought to be unexcitable cells that simply provided a scaffold to help organize neurons.[35] However, it is now known that astrocytes perform many more tasks. These functions include release of molecules that guide axon growth and the formation of glial boundaries,[25] removal of excess glutamate from neuronal synapses and regulation of synapses using glutamate,[36] regulation of blood flow in the brain's cerebral region by relay of neuronal signals to blood vessels,[37] reactivity to CNS trauma,[19] and control of the blood brain barrier,[25] to name a few. Astrocyte function differs from other brain cells, most notably neurons, in that astrocytes do not generate action potentials, which is the reason they were long thought to be unexcitable cells.[35] It is now known, however, that astrocytes can receive and deliver messages to and from surrounding cells. Astrocyte excitation is not generated via electrical means as in neurons but rather via chemical signals from neurons or by spontaneous release of internal calcium due to activation of Ins(1,4,5)P3 receptors.[35] Additionally, astrocytes are connected with each other via gap junctions, allowing them to move and redistribute harmful ions over long distances.[24]

As mentioned previously, astrocytes become reactive and undergo changes in morphology, expression of certain proteins, and function in response to CNS trauma. There is a significant up regulation of GFAP in response to CNS trauma, which is the

major marker of reactive astrocytes.[24] However, it is unclear if astrocyte reactivity is beneficial in the case of CNS trauma or if it actually causes more damage than good. For instance, in the case of CNS trauma there is a substantial release of glutamate from neurons,[38] which is normally taken up by astrocytes. However, activated astrocytes exhibit a highly reduced and sometimes even reversed uptake of glutamate, which can lead to more neuron damage.[39] Activated astrocytes have also been seen to produce nitric oxide, which is a molecule toxic to neural cells.[40] Studies have shown benefits of astrocyte activation as well, particularly the up regulation of GFAP and the presence of vimentin, another important astrocyte intermediate filament. Pekny et al. showed that in mice lacking both GFAP and vimentin CNS trauma resulted in reduced glial scar formation, a major part of the CNS healing process, as well as increased bleeding.[41] It has also been shown that the glial scars formed by reactive astrocytes can act to surround damaged tissue, helping to localize inflammation and allowing for faster reduction and healing of inflammation. The presence of reactive astrocytes has also been shown to aid in blood-brain barrier repair, reduce toxic edema, and protect other CNS cells.[40] Clearly, astrocyte reactivity is a mechanism that has both positive and negative effects on the CNS.

2.2: Differences Between GBM and Normal Brain Tissue

As mentioned previously, the GBM tumor is a form of astrocytoma, meaning that GBMs arise from astrocytes. Because these GBMs are derived from astrocytes, it is of

interest to identify major differences between these cell types and differences between GBMs and other normal brain tissue. Gingras et al. showed that GBM cells exhibit higher expression of several cell adhesion molecues (CAMs) than normal brain tissues. These CAMs included the integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$, the immunoglobin gene superfamily CAMs ICAM-1 and LFA-3, and the CAM known as TACA.[42] Additionally they exhibit major differences in expression of several genes and proteins between GBMs and astrocytes.[18] For example, GBMs express significantly higher levels of matrix metalloproteinase-2 (MMP-2) and significantly lower levels of GFAP than their astrocyte counterparts. Further, astrocytes express significantly higher levels of tissue inhibitor of MMP-2 (TIMP-2), secreted protein acidic and rich in cysteine (SPARC), and connexin 43 (CX43).[18] A more comprehensive database developed by Lal et al., comparing the expression of genes between GBMs and normal brain white matter, found 471 different genes that had at least a 5-fold difference in expression. Classes of genes investigated by this work included genes involved with angiogenesis, which were increased in GBMs, genes involved with neurotransmission, which were decreased in GBMs, and genes involved in metaplasia, which were increased in GBMs, in addition to many others.[43]

Astrocytic tumors do not start as GBMs, they progress through different levels, referred to as grades, of tumor. The first level is grade I, referred to as a pilocytic astrocytoma, seen mostly in juveniles. The pilocytic astrocytoma is very distinct from astrocytoma grades II-IV in terms of age of those affected by it, genetic origin, behavior, and location in the brain.[44 45] The tumor then progresses to grade II, called a low grade

astrocytoma. Further tumor progression leads to a grade III tumor, known as an anaplastic astrocytoma. The final level of astrocytoma progression is grade IV, the GBM.[46] It is important to look at differences in the different grades of astrocytoma as well as astrocytoma differences from astrocytes (see Figure 3). The pilocytic astrocytoma is benign and tends to show an up regulation of the p53 protein in some cases[44] and a loss of portions of chromosome 17.[45] Low grade astrocytomas commonly exhibit mutation of the p53 gene (24% incidence), sometimes accompanied by a mutation in chromosome 17p. Transition from grade II to grade III astrocytoma is marked by an increase in anaplasia, cell proliferation, irregularity of cell nuclei, and cellularity.[44] Additionally, grade III astrocytomas can exhibit p53 mutations (34 percent incidence), deletion of multiple tumor suppressor 1 (44% incidence), and mutation of chromosome 19q (44% incidence).[44] Progression to grade IV glioblastoma is marked by continued increase of cell proliferation, growth of tumor blood vessels, continued mutation of p53, amplification of cyclin dependent kinase 4 (CDK4, 76% incidence), and loss of chromosome 10 (60% incidence).[44] A glioblastoma that has progressed from a grade III astrocytoma is termed a secondary glioblastoma. It is possible and more common, however, for astrocytes to turn directly into glioblastomas. These glioblastomas are termed primary glioblastomas and are seen to have higher EGFR amplification and p16 deletion but lower p53 mutation than secondary GBMs.[47]

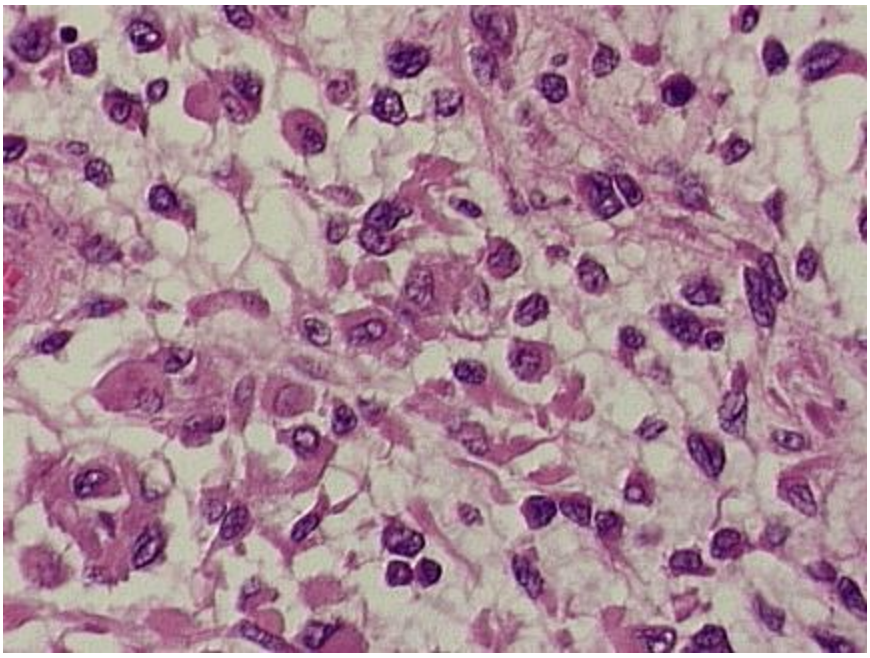

Figure 2: GBM in Culture (figure taken directly from http://radiology.uchc.edu/eAtlas/CNS/1833.htm)[48]

2.3: Composition of Brain ECM

The interactions between the extracellular matrix of the brain and glioblastoma cells greatly influence GBM migration, including GBM attachment to and degradation of ECM molecules followed by GBM migration.[24] Studies have shown that normal brain ECM differs from ECM in the presence of brain tumors as a result of modification by brain tumor cells.[25]

The ECM has many important functions in the normal brain, including modulation and guidance of brain cell migration and growth and axonal growth in the developing brain, brain tissue repair, and modulation of the activity of certain growth factors.[49] The ECM of the brain differs greatly from the ECM of other body tissues in

that it largely lacks the proteins that are most prevalent in ECM of the rest of the body.[50] The ECM of the normal brain is mainly composed of hyaluronan, proteoglycans (more than 25 thought to be present), tenascin-C (mostly present in white matter), and thrombospondin. Hyaluronan is the most prevalent component of the brain ECM and allows for large expansion of the brain extracellular space. [49] The lectican family of proteoglycans is thought to be the major proteoglycan player in the brain ECM, as it has the ability to bind to both tenascin-C and hyaluronan. Lecticans form a complex with these other two molecules, which is believed to be the major assembly of the ECM.[51] Another important molecule in the brain ECM is Reelin. Reelin plays a major role in migration of neurons, organization of the brain during development, and regulation of receptors which control calcium influx in postsynaptic neurons.[52] In addition to the aforementioned components, brain ECM also contains small amounts of proteins more typically found in the ECM of other parts of the body, including collagen, laminin, and fibronectin. However, these components are only present in the vascular regions of the brain, causing the brain to be much softer than most organs.[49]

The ECM of glioma cells has been shown to contain mostly tenascin (upregulated in glioma ECM), fibronectin, laminin, vitronectin, and a few different kinds of collagen.[53] Glioma ECM also includes osteopontin, SPARC, brain enriched hyaluronan binding proteoglycan (BEHAB, also known as brevican), and sees a four-fold increase in hyaluronan.[49] The presence of BEHAB facilitates invasion and migration of brain tumor cells. [49] The increase in tenascin has also been shown to promote brain tumor

proliferation and migration.[54] Glioma cells interact with and adhere to the ECM through the use of integrins and CD44, a hyaluronan receptor. Integrins are transmembrane molecules that give the glioma cells a physical connection to the ECM, communicate signals across the membrane, and recruit proteases to the leading edge of the cell that can break down the surrounding ECM, clearing a pathway for the cell to migrate. The major proteases in ECM degradation by glioma cells are the family of matrix-metalloproteinases (MMP). Studies have shown a large up regulation of MMPs, particularly MMP-2, in glioma cells versus normal astrocytes[18] and MMP inhibitors have been shown to significantly decrease glioma invasion.[25] CD44 is a family of cell adhesion molecules that bind to hyaluronan and have been implicated as a major component for glioma migration and invasion.[55]

2.4: Biology of GBM migration

Because of the problems and dangers caused by the migration of GBM cells away from the primary tumor mass, it is important to understand the physical mechanisms and molecular pathways behind it. The physical mechanism of glioblastoma migration begins with breakdown of the ECM at the leading edge of the migrating cell, as described above. The cell then uses the surrounding ECM molecules for traction to allow movement. It does this by developing protrusions made of actin and other proteins, which then adhere to ECM molecules. This adhesion is followed by contraction of the cell cytoskeleton to propel the cell forward.[25] This ECM guided

migration takes place along the outside of white matter tracts and blood vessels of the brain,[56] making these an important component of any study of GBM migration.

There are many molecular signaling pathways that have been implicated in GBM migration. One pathway that is known to be involved in cell migration of both normal and tumor cells is the focal adhesion kinase (FAK) pathway activated by autophosphorylation of FAK residue Y397.[57] FAK is a tyrosine kinase found in the cytoplasm that functions by recruiting certain molecules to integrin receptors. This forms a signaling pathway to communicate signals from the ECM to the cell cytoskeleton.[57] Because of its known involvement in migration of many types of cells,[58] FAK has been of interest in the study of GBM migration. FAK up regulation has been seen in invasive astrocytomas such as GBM, suggesting an important role for FAK in GBM migration.[59] Wang et al. looked at the effects of overexpression of FAK on migration and growth of astrocytoma cells and found that cells overexpressing FAK saw a 2 to 3-fold increase in migration *in vitro* and a 1.6 to 2.8-fold increase in total number of tumor cells *in vivo*.[60] Jones et al. looked at FAK mediated migration by stimulation of epidermal growth factor receptor (EGFR) by comparing migration of normal GBM cells with that of GBM cells transfected with a focal adhesion targeting (FAT) domain to inhibit FAK. GBM migration was significantly reduced in FAK inhibited cells, further suggesting an important role for FAK as a major pathway in GBM migration.[61] Kolli-Bouhafs et al. treated GBM cells with Thymoquinone (TQ) and looked at effects of TQ on GBM viability and migration as well as GBM expression of FAK. GBM cells treated with

TQ were shown to have a significant reduction in viability and migration in addition to a significant reduction in FAK expression, suggesting a correlation between FAK expression and GBM migration and viability.[62]

Whereas the data generated from these studies discussed above indicate a major role for FAK in GBM migration, there has also been contrasting work. Studies by Lipinski et al. have seen overexpression of FAK leading to decreased GBM migration and increased expression of another tyrosine kinase, proline-rich tyrosine kinase 2(Pyk2), correlating to increased GBM migration. These studies have also found that over expression of FAK stimulates cell proliferation and cell cycle progression.[10 63] These data would suggest that Pyk2 may also be a major player in GBM migration. Pyk2 is a member of the FAK sub-family and is activated by an increase in intracellular calcium levels.[64] Overexpression of Pyk2 has been shown to induce apoptotic cell death in fibroblasts of rats and mice.[65] Gutenberg et al. looked at the expression of both FAK and Pyk2 and how expression of these kinases correlated with increasing astrocytoma grade. They found a significant correlation between increasing expression of FAK and Pyk2 and increasing astrocytoma grade. They also found a much more frequent and higher expression of Pyk2 than FAK in all astrocytoma grades.[66] Loftus et al. substituted residues on the FERM domain of Pyk2 to inhibit phosphorylation of Pyk2. This in turn reduced migration of glioma cells, further indicating Pyk2 as an important aspect of migration.[67] Taken together, all of these studies indicate that both FAK and Pyk2 are

major regulators of glioma migration and may be potentially useful therapeutic targets in the future.

In addition to FAK and Pyk2, other proteins are also thought to play a role in GBM migration. For example, the activation of members of the Rho family of GTPases, in particular Rho and Rac, leads to attachment of glioma cells to a surface by creation focal adhesions and formation of cell processes, which allows for glioma migration.[68] [69] Zohrabrian et al. investigated the effects of inhibition of the Rho pathway via inhibition of Rho-associated protein kinase (ROCK) on glioblastoma migration. They found that inhibition of ROCK led to a significant decrease in GBM migration. [69] Zhai et al. invasion and migration of GBM cells treated with radiation and found that radiation treated cells had increased invasiveness and migration speed. This resulted from Rho activation via the Phosphoinositide 3-Kinase Pathway (PI-3K). They also saw a more moderate decrease in migration under normal physiological conditions without radiation when PI-3K was inhibited, although they saw no difference when Rho was inhibited.[70] The PI-3K pathway, which is activated downstream of FAK,[71] has also been investigated as a major pathway involved in GBM migration under non-radiative physiological conditions. Chandrasekar et al. looked at the effects of downregulating urokinase-type plasminogen activator (uPA) on PI-3K activation and on GBM migration. They found that downregulation of uPA decreased PI-3K activation and GBM migration, suggesting a correlation between PI-3K activation and GBM migration. [72] Another set of molecules that is highly expressed in GBMs and has been implicated as playing a role in GBM

migration is the Src family of protein kinases.[73] Src has been shown to work in conjuction with FAK to promote formation of protrusions of cell membranes and to promote cell migration.[57] Studies have shown that migration of GBM cells can be promoted by Src regulation[74] and that blockage of Src activity can decrease GBM invasion.[75] Clearly there are many molecules and pathways involved in GBM migration and invasion, many of which seem to work in conjunction with one another to promote migration and invasion. This provides many potential therapeutic targets to inhibit GBM migration but also raises much uncertainty about which molecules and pathways are most important in GBM migration.

2.5: Other Normal Brain-GBM Co Culture Models

Substantial previous work has been performed to study the interactions of normal brain cells and GBM cells in co culture. These studies have investigated expression of certain genes by both cell types in co culture, migration of glioma cells and normal brain cells, and other interactions including effects of gap junctions and effects of conditioned media. These studies were all performed in a variety of different environments, each of which has its positive and negative aspects.

Early co culture studies investigated GBM-astrocyte interactions in 2D tissue culture dishes. These studies found interactions occurring between the two cell types. Glioma cell invasion into rat brain aggregates and tissue was investigated, with invasion being seen to progress over 10 days until finally destroying the aggregates. Viability of

brain aggregates grown in glioma conditioned media was also investigated, with inconsistent results being found.[76] Glioma cells were also seen to influence GFAP expression of astrocytes, causing GFAP upregulation,[77] as well as cause an increase in production of the active form of metallomatrix proteinase 2 (MMP-2), a gene indicated in increasing glioma invasion, by astrocytes in coculture.[78] In recent years co culture studies have been performed in more physiologically relevant models approaching three dimensions. Gagliano et al. performed a co-culture of astrocytes and glioma cells in a 6 well transwell insert system to look at changes in expression of certain genes and proteins that play important roles in glioma invasion. In particular, they looked at GFAP, MMP-2, TIMP-2, transforming growth factor-β1 (TGF-β1), SPARC, and CX43. They saw increases in expression of GFAP, MMP-2, and SPARC protein, decreases in expression of TIMP-2, SPARC mRNA, and CX43, and no change in TGF-β1 expression in co cultures versus cultures of astrocytes only.[18] Other studies have established the effects of gap junctions formed between glioma cells and astrocytes, finding that inhibition of glioma-astrocyte gap junctions decreased migration.[79]

Clearly astrocyte-glioma interactions have been studied in some detail previously and important findings have been made. However, there are shortcomings with many of the systems used in the discussed experiments. Several of them were performed on 2D tissue culture dishes, which while useful do not mimic the environment encountered by these cells *in vivo*. Experiments performed in a transwell migration chamber are somewhat three dimensional, making them more like what is seen *in vivo*. Similarly,

experiments performed on astrocyte monolayers and on collagen type IV were more physiologically relevant. However, they still do not closely mimic the topography of the brain in terms of presence of physiologically relevant structures (i.e. blood vessel periphery and white matter tracts) and still do not allow 3D movement of cells. Additionally, Collagen type IV does not provide potential soluble cues released by other living, non-transformed brain cells. Electrospun fibers provide an environment that more closely mimics the topography of the major brain structures by which GBM migration occurs, and the addition of astrocytes to this environment provides yet another component that makes this system more biomimetic.

Chapter 3: Experiments and Results

It is clear that the GBM is a highly dangerous tumor that is still very poorly understood. Thus, it is important to gain a better understanding of this tumor, its interactions with the brain, and its migratory behavior to find better ways to treat it. We investigated GBM migration and astrocyte GFAP expression in a three dimensional, physiologically relevant environment in hopes of elucidating the effects of GBMs on astrocyte behavior and vice versa. We performed co-culture studies to look at GFAP expression in astrocytes and the possible causes of GFAP up regulation in presence of GBM cells. We have also performed co-culture studies to look at the effect the presence of astrocytes has on GBM migration and the possible causes of these effects.

3.1: Materials and Methods

3.1.1: Culture of Rat Astrocytes

Cortical rat astrocytes (Invitrogen) were grown in plastic tissue culture dishes in medium consisting of 84% Dulbecco's Modified Eagle Medium (DMEM) High Glucose 1X (Invitrogen), 15% Fetal Bovine Serum (FBS), and 1% penicillin/streptomyocin (P/S), with medium being changed every 3-4 days per Invitrogen protocol. Upon reaching full confluence, astrocytes were dissociated from dishes using StemPro Accutase (Life Technologies), centrifuged at $250 \times g$ (5 mi), and then either seeded onto a new culture

dish at an approximate density of 2 x 10^4 cells/cm^2 or used for cell culture experiments on fibers.

3.1.2: Culture of Glioblastoma Cells

The glioblastoma cells used in these experiments were derived from a patient with glioblastoma multiforme at the Ohio State University with informed consent under human IRB protocol 2005C0075.[80] OSU2 cells were cultured on plastic tissue culture dishes in medium consisting of 89% DMEM/F-12 (Ham) 1X (Life Technologies), 10% FBS, and 1% P/S, with media being changed every 2-3 days. Cells were passage upon reaching confluency, and then either seeded onto a new culture dish at an approximate density of 2 x 10^4 cells/cm^2 or used for experiments.

3.1.3: Co Culture of Rat Astrocyte and OSU2 Cells for GFAP Expression Investigation:

When both rat astrocyte and OSU2 cells reached confluence, both cell types were detached and resuspended in fresh medium as described previously. Astrocytes were then labeled with Cell Tracker Green CMFDA (5-Chloromethylfluorescein Diacetate) fluorescent dye (Invitrogen) and OSU2 cells were labeled with Cell Tracker Red CMPTX fluorescent dye (Invitrogen). After labeling with dye all cells were resuspended in rat astrocyte media (84% DMEM, 15% FBS, 1% P/S). Rat astrocyte media was tested as a growth media for OSU2 cells and it was found that OSU2 cells were able to survive and grow normally in rat astrocyte media. In addition to co-

culture, GFAP expression was investigated in astrocytes cultured in media conditioned by OSU2 cells. OSU2 conditioned media was prepared by seeding OSU2 cells in fiber mat wells at a count of 800 cells per 100 µL of media for 48 hours. All experiments took place in 96 well plates with each well consisting of an aligned electrospun fiber mat (Figure 3, properties in Table 1) attached to the bottom of the well. Experimental conditions are shown in Table 2.

Table 1: Properties of electrospun PCL fibers used in this work

	Fiber Diameter (µm)	Fiber Density (fiber/ mm^2)	Fiber Modulus (MPa)
PCL Fibers	1.17±0.26	24938±5312	~8

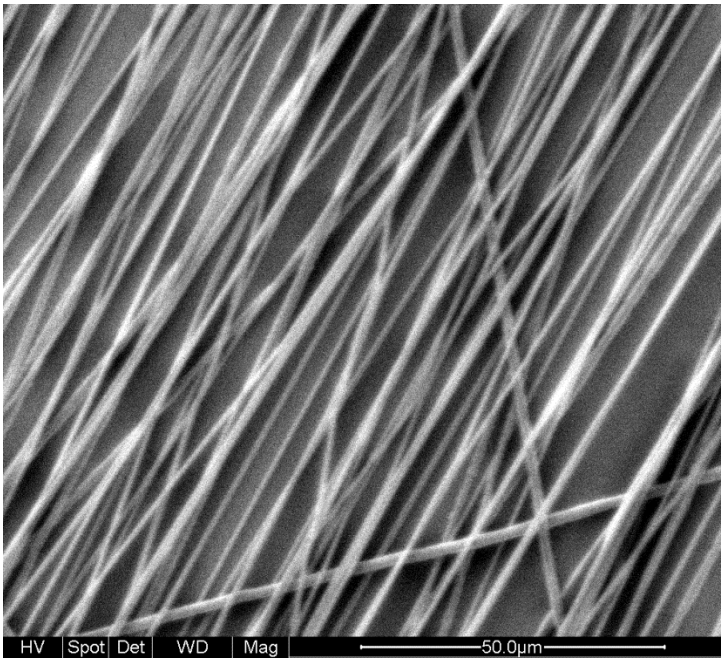

Figure 3: SEM of electrospun fiber mat

Table 2: Experimental conditions for co culture GFAP experiments

Condition	Rat Astrocyte (Stained Green) Count	OSU2 (Stained Red) Count	Media Used	Ratio
Condition 1	8000	800	Rat Astrocyte Normal	10:1
Condition 2	8000	80	Rat Astrocyte Normal	100:1
Condition 3	8000	8	Rat Astrocyte Normal	1000:1
Condition 4	8000	N/A	OSU2 Conditioned	0
Condition 5	8000	N/A	Rat Astrocyte Normal	0

After the proper volume of media was added to each well to give the proper numbers of each cell type, fresh rat astrocyte media was added to the wells as needed to bring the total volume of media in each well up to 100 µL. Cells were then allowed to attach and culture for 48 hours in a 37^{o}C , 5% CO_2 incubator.

After 48 hours of incubation, plates were removed from the incubator and media was aspirated. Cells were then washed with PBS (2X), fixed in paraformaldehyde solution (40 mg/mL in PBS) for 20 minutes, washed again with PBS (2X), extracted using a solution of Triton-X 100 (2 µL/mL in PBS) in PBS for 15 minutes, washed again with PBS (2X), and incubated in blocking solution (30 mg/mL of Bovine Serum Albumin (BSA) in PBS). Blocking solution was then removed, cells were washed once with PBS, 50 µL/well

of a primary antibody solution (30 mg of BSA and 5 µL of Anti-GFAP antibody - Astrocyte Marker (Abcam product number ab7260) together in 1 mL of PBS) was added, and cells were stored in a 4 degree Celsius refrigerator overnight. Following this the primary antibody solution was aspirated, cells were washed twice with PBS, and a secondary antibody solution (30 mg BSA and 2 µL of blue goat anti-mouse secondary antibody stain (DyLight 405 AffiniPure Goat Anti-Rabbit IgG (H+L) (min X Hu,Ms,Rat Sr Prot), product number 111-475-144, Jackson ImmunoResearch)) together in 1 mL of PBS) was then added to each well and the plate was incubated for 20 minutes. After 20 minutes the stain was aspirated and cells were washed twice with PBS and imaged with epifluorescence microscopy (Olympus IX 71) using appropriate fluorescent channels.

3.1.4: Analysis of GFAP Expression

As mentioned in the protocol above, rat astrocytes were stained green with Cell Tracker Green CMFDA dye, OSU2 cells were stained red with Cell Tracker Red CMPTX dye, and GFAP was stained blue with DyLight 405. GFAP expression was quantified by determining blue fluorescent intensity of cells identified as rat astrocytes via their green fluorescence. Fluorescent intensity was determined by analyzing blue channel images using ImageJ image analysis software (downloaded for free from National Institutes of Health, http://rsbweb.nih.gov/ij/download.html). Blue channel images were first pulled up and an average background fluorescence of each image was determined. This was accomplished by first using the ImageJ oval drawing tool to draw an oval at ten separate

points directly next to cells that were to be analyzed. ImageJ was then used to measure the mean fluorescence of each oval, and the average of the mean fluorescence values of the ovals was found, providing an average background intensity value. Blue images were then compared to green channel images to identify rat astrocytes on the blue channel images. The ImageJ freehand drawing tool was then used to trace around individual rat astrocyte cells and ImageJ was used to measure the area and integrated density (the sum of all pixel values in the selection) of the selection. A corrected total cellular fluorescence (CTCF) was then calculated for each cell by subtracting the product of the area of the cell and the mean background fluorescence from the integrated density of the cell as described elsewhere.[81] After cell fluorescence analysis was completed, fluorescent values across each condition were tested for statistical difference via ANOVA and student's t-test performed in the statistical software package JMP Pro 9.

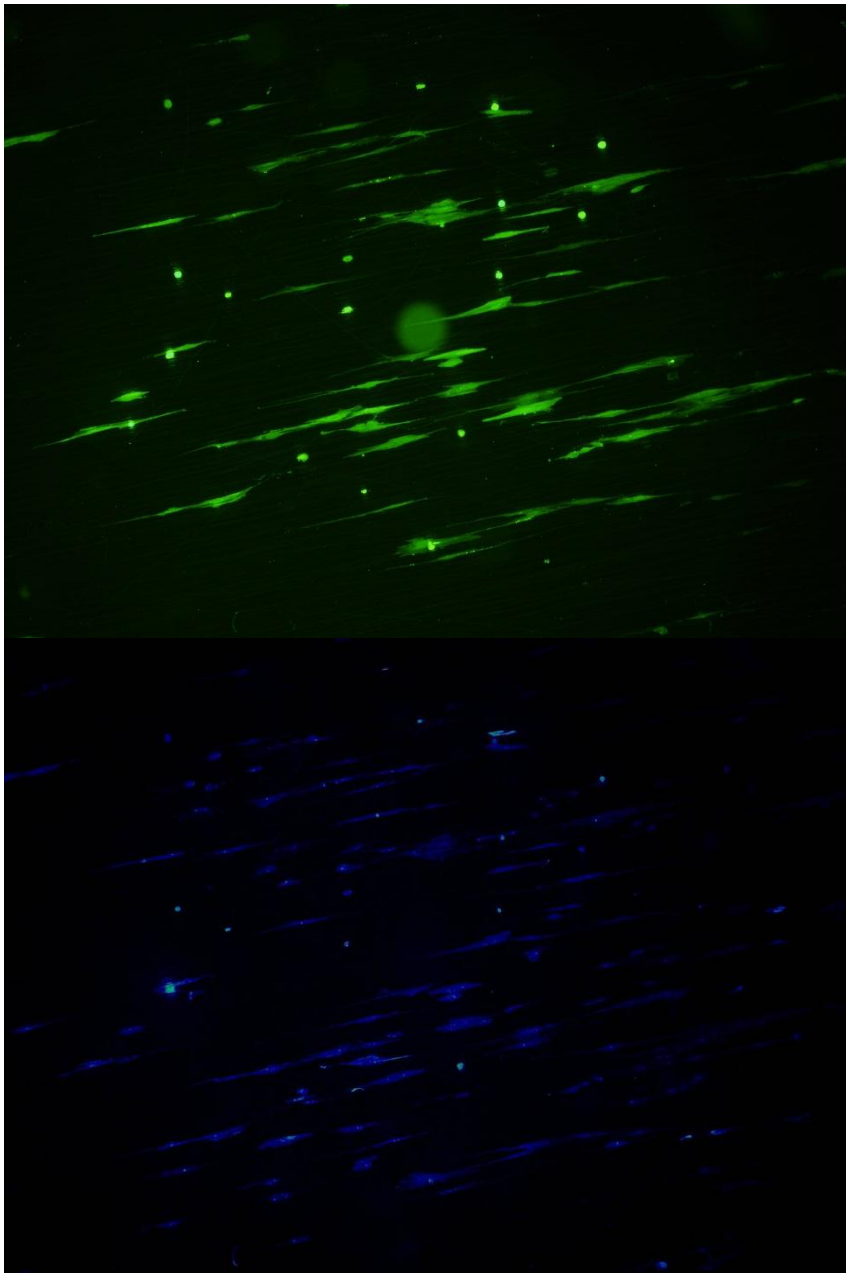

Figure 4: Rat Astrocytes and GFAP Expression

3.1.5: Co Culture of Rat Astrocyte and OSU2 Cells for OSU2 Migration Investigation

Co culture of rat astrocytes and OSU2 cells for experiments investigating migration of OSU2 cells were performed in the same manner as described in section

3.1.3 with a few notable exceptions. In this case the colors used to dye cells were reversed. OSU2 cells were stained using Cell Tracker Green CMFDA and rat astrocytes were stained using Cell Tracker Red CMPTX. Also, cell concentrations used varied. In particular, in this experiment GBM cell numbers were held constant while astrocyte numbers varied. This was a reversal of the previous experiment in which astrocyte numbers were held constant while GBM numbers varied. Experimental conditions for this experiment are shown in table 2.

Table 3: Experimental conditions for OSU2 migration investigation in co culture system

Condition	Rat Astrocyte (Stained Red) Count	OSU2 (Stained Green) Count	Media Used	Ratio
1	15000	1000	Normal Rat Astrocyte Media	15:1
2	10000	1000	Normal Rat Astrocyte Media	10:1
3	5000	1000	Normal Rat Astrocyte Media	5:1
4 (Control)	N/A	1000	Normal Rat Astrocyte Media	N/A

After initial seeding in each well, cells were incubated for 5 hours to allow for cell attachment. After 5 hours, media was replaced and plates were incubated a weather station that kept the environment constant at 37 degrees Celsius and 5% CO_2. After a 3

hour period to allow for environment adjustment, an epifluorescent microscope (Olympus IX-71) was used to identify 2-3 positions in each well that were tracked over a period of 12 hours by time lapse microscopy. After all desired positions were identified, the microscope and camera were automated to collect 37 sets of Z-stack images of each well position, equivalent to one set every 20 minutes. After time lapse had completed, images were compiled into movies.

3.1.8: Preparation of Conditioned Media for Migration Experiments

OSU2 migration was also investigated in rat astrocyte conditioned media to identify possible influence of stable soluble factors secreted by normal rat astrocytes. For initial experiments conditioned media was obtained from rat astrocyte cultures on polystyrene tissue culture plates. Upon reaching approximately 90% confluence, rat astrocytes were fed fresh rat astrocyte media and allowed to incubate for 48 hours. After 48 hours, conditioned media was collected and used for migration studies. In later experiments, conditioned media was obtained from rat astrocytes being grown on electrospun nanofibers. Rat astrocytes were seeded on electrospun fiber mats in 96 well plates at a density of 10000 cells per well and incubated in 100 µL of rat astrocyte medium. Culture time was also tested as a variable in this experiment, so cultures lasted for set times of either 24 hours or 48 hours. After the proper amount of time had elapsed conditioned media was removed and stored in sterile 15 mL centrifuge tubes

until use for an experiment (Figure 3). After conditioned media was created, migration experiments were performed as described above tracking the migration of OSU2 cells in conditioned media versus a control group, OSU2 cells in normal, unconditioned rat astrocyte media.

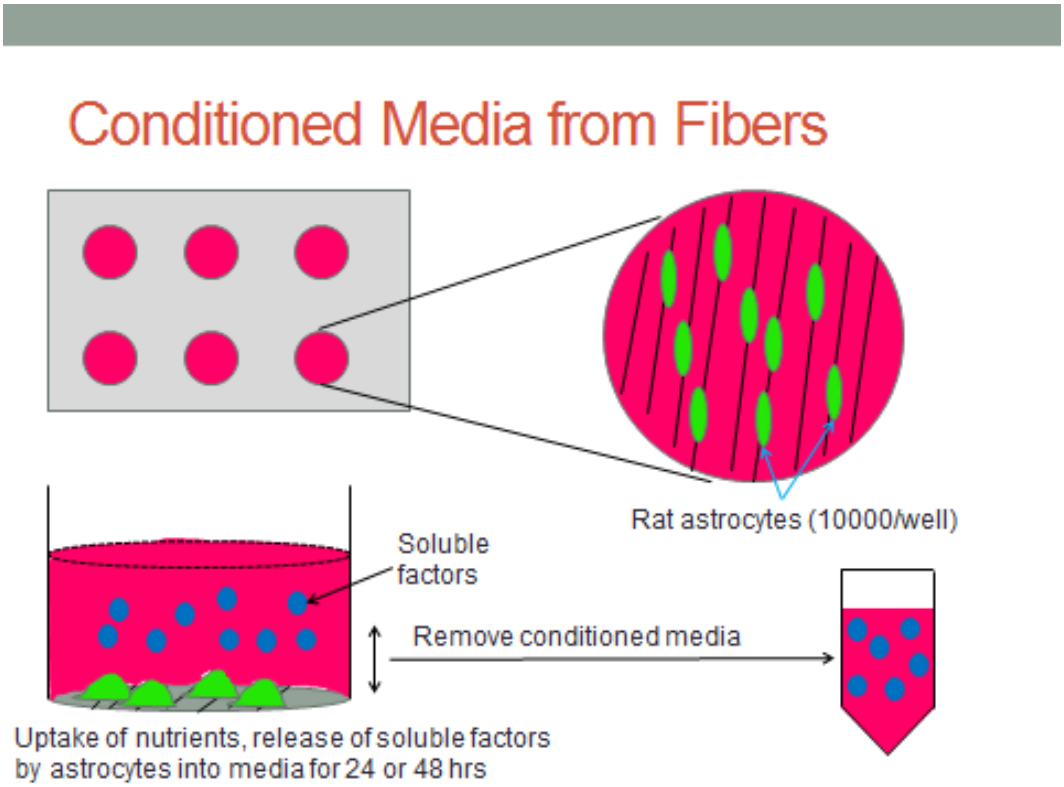

Figure 5: Schematic of process of obtaining conditioned rat astrocyte media

3.1.9: OSU2 Migration in Presence of Fixed Astrocytes

To investigate the effects of the physical presence of astrocytes on OSU2 migration, experiments were performed by fixing astrocytes to wells. Astrocytes were added to 96 well fiber plates at counts of either 10000 or 15000 cells. Astrocytes were

allowed 6 hours to adhere to plates, after which media was removed and cells were washed twice with sterile PBS. A fixing solution consisting 40 mg/mL paraformaldehyde in PBS was prepared as described previously. The fixing solution was then sterilized by passing it through a 22 µm syringe filter (Fisher Scientific). This fixing solution was then added to each well of cells for 20 minutes, after which the solution was removed and the cells were again washed twice with PBS. OSU2 cells were then added to the wells and their migration was tracked as described previously. (Figure 4)

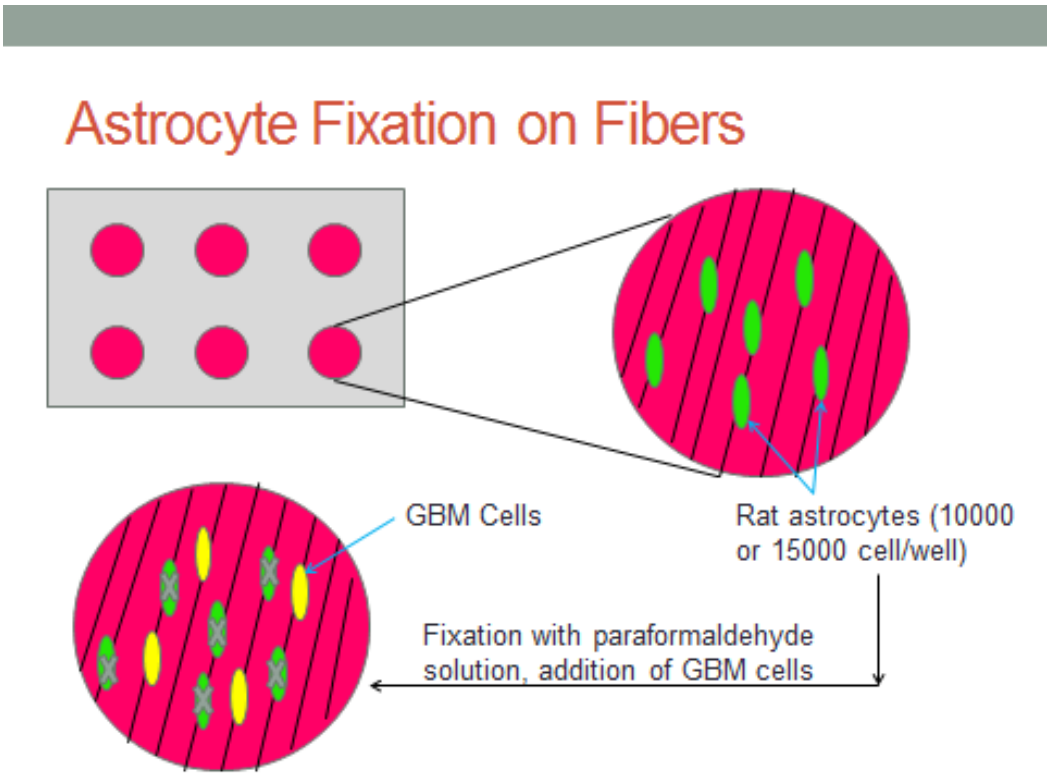

Figure 6:Schematic of process for fixing astrocytes on fibers

3.1.10: OSU2 Migration on Astrocyte ECM Deposited Fibers

To investigate the effects of astrocyte ECM on OSU2 migration, astrocytes were cultured on electrospun fibers and then removed while keeping ECM deposited on the fibers intact (verified by scanning electron microscope images). Astrocytes were seeded on 96 well fiber plates at a density of 10000 cells per well and allowed to adhere for set times of either 24 hours or 48 hours. After the proper time had elapsed, media was removed and cells were washed once with 100 µL of sterile PBS per well. One hundred µL StemPro Accutase was then added to each well and astrocyte detachment was monitored by counting cells present in the StemPro with a hemocytometer. A count of approximately 70% cell removal or more was considered the stopping point for detachment. This was done because of the possibility of some cells getting stuck to the pipette and thus not getting counted and to reduce the likelihood of causing ECM detachment as well. To ensure cell removal as well as ECM retention, scanning electron microscopy (SEM) images of samples were collected. After StemPro was removed, cells were washed three times with sterile PBS. OSU2 cells were then stained green, seeded at a density of 1000 cells per well, and migration was tracked via time lapse microscopy as described previously. (Figure 5)

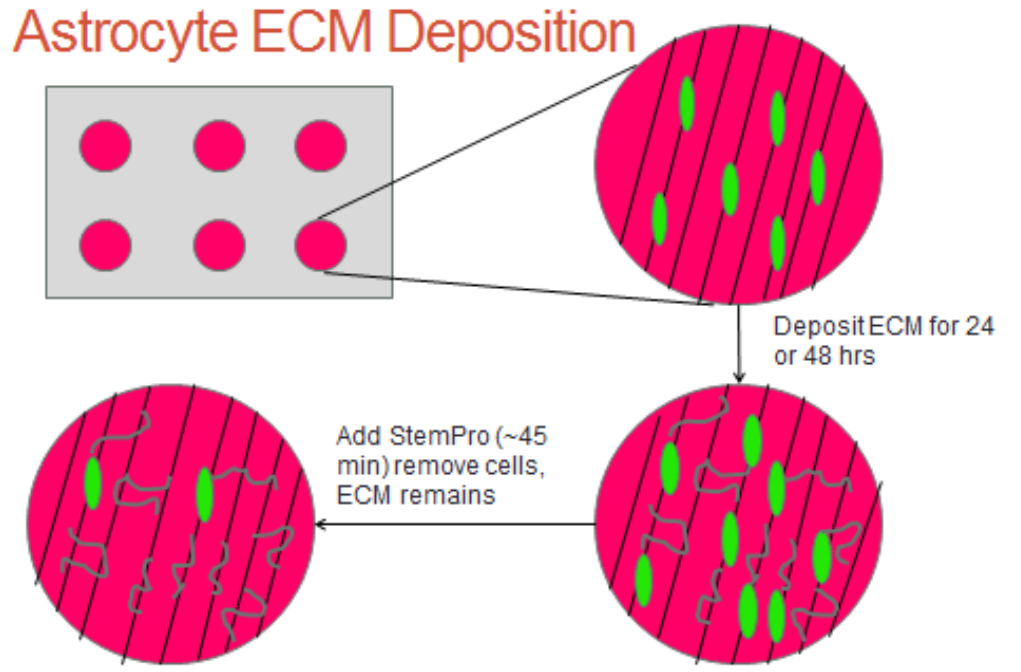

Figure 7: Schematic of process ECM deposition by astrocytes and astrocyte removal

3.1.11: Dehydration of Astrocyte ECM Samples for SEM

To verify that ECM was deposited by astrocytes for the experiment described in section 3.1.10, some samples were imaged using scanning electron microscopy (SEM). Astrocytes were seeded and removed as described in section 3.1.10. Samples to be used for SEM were then washed in sterile PBS, followed by deionized (DI) water for 1 hour on a rocker. After washing was completed, DI water was removed and samples were punched out of 96 well plates using a biopsy punch. Samples were then placed into 0.6 mL centrifuge tubes and fixed by flash freezing in approximately 100 mL of liquid nitrogen. After liquid nitrogen had evaporated, centrifuged tubes were closed

and placed in a lyophilizer overnight to dehydrate samples. Dehydrated samples were then coated with gold in a plasma sputtering machine and images were collected with the SEM (Quanta 200).

3.1.12: Analysis of OSU2 Migration

After time lapse microscopy of cells had completed, images were stacked into 37 frame movies. Individual migrating cells were tracked frame by frame using the MtrackJ plugin. MtrackJ gave results for migration distance in pixels, which was then converted to micron/hr using a conversion factor of 0.133. This conversion factor was found using an image of a hemocytometer of known dimensions at the same magnification used for migration tracking and determining micron per pixel. This number was then divided by 12 to find micron/pixel/hour. Migration of cells under each condition was then analyzed using the statistical software JMP Pro 9 by way of student's T test and Dunnet's control method. Data was pooled from multiple experiments involving the same conditions to give a larger sample size and to provide more significant results.

3.2: Results

3.2.1: GFAP Expression of Rat Astrocytes in Co Culture with GBM

Co culture conditions were performed on electrospun fiber mats at ratios of 10:1, 100:1, and 1000:1 rat astrocyte:GBM (table 1) and GFAP expression of astrocytes was quantified using immunofluorescence. GFAP values for co cultures were then

compared to GFAP expression of astrocytes in culture alone. As the ratio of astrocytes to GBM cells decreased, expression of GFAP increased. Corrected total cellular fluorescence (CTCF) values (described in section 3.1.4) were the quantification of GFAP expression. These were then divided by the mean of the control condition to normalize values. All co culture conditions showed a significant increase in astrocyte GFAP expression versus the control, with the 10:1 ratio co culture showing a nearly two-fold increase versus the control (Figure 8). The 10:1 ratio co culture condition also exhibited a significant increase in GFAP expression over the 100:1 and 1000:1 ratio conditions.

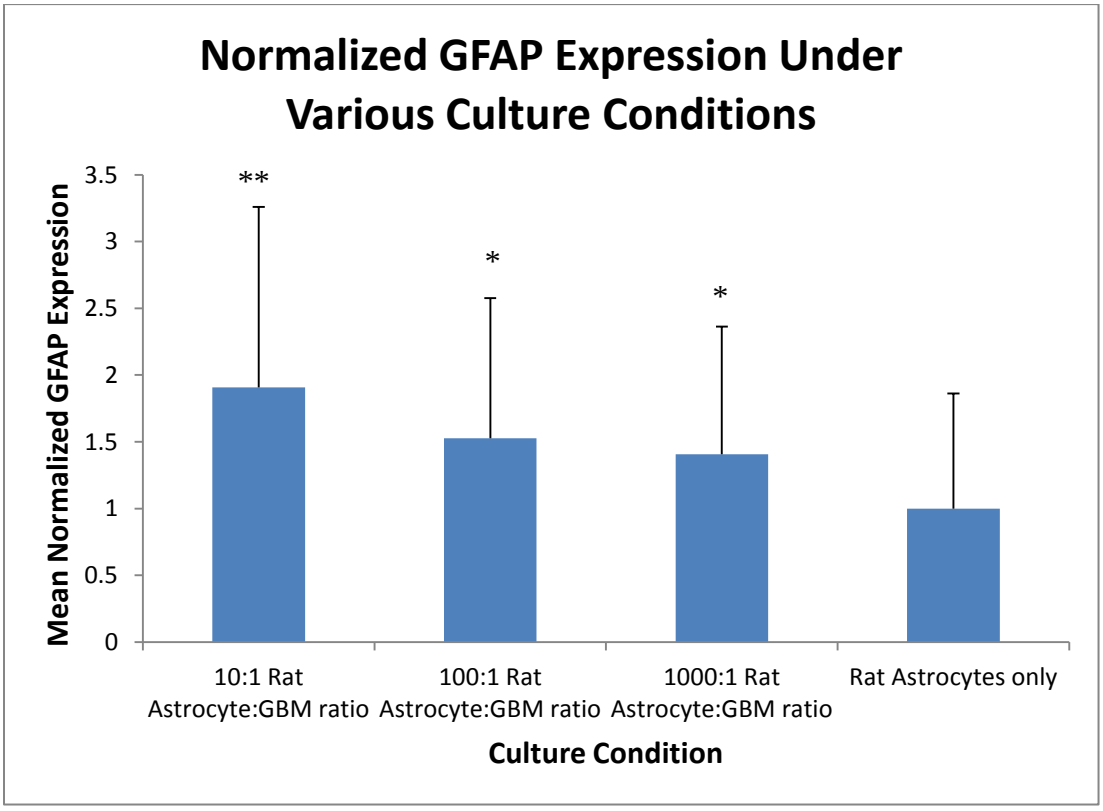

Figure 8: Normalized astrocyte GFAP expression in various conditions, n≥166 cells analyzed per condition, p-value≤0.005, * denotes significant difference from control (rat astrocytes only), ** denotes significant difference from control and other two conditions,

3.2.2: Rat Astrocyte GFAP Expression in GBM Conditioned Media

Several possible causes of the evidenced GFAP increase were identified and investigated. These causes included direct contact with a protein or surface marker, expressed on either the GBM cell surface or in its ECM, or by a soluble molecule released into solution. To evaluate the possibility of GBM released stable soluble factors stimulating GFAP increase, GBM cells were grown on fiber mats for 48 hours, after which media was collected and saved. Rat astrocytes were then grown on fiber mats in normal media and in GBM conditioned media. No difference in expression of GFAP was detected between the two conditions (Figure 9).

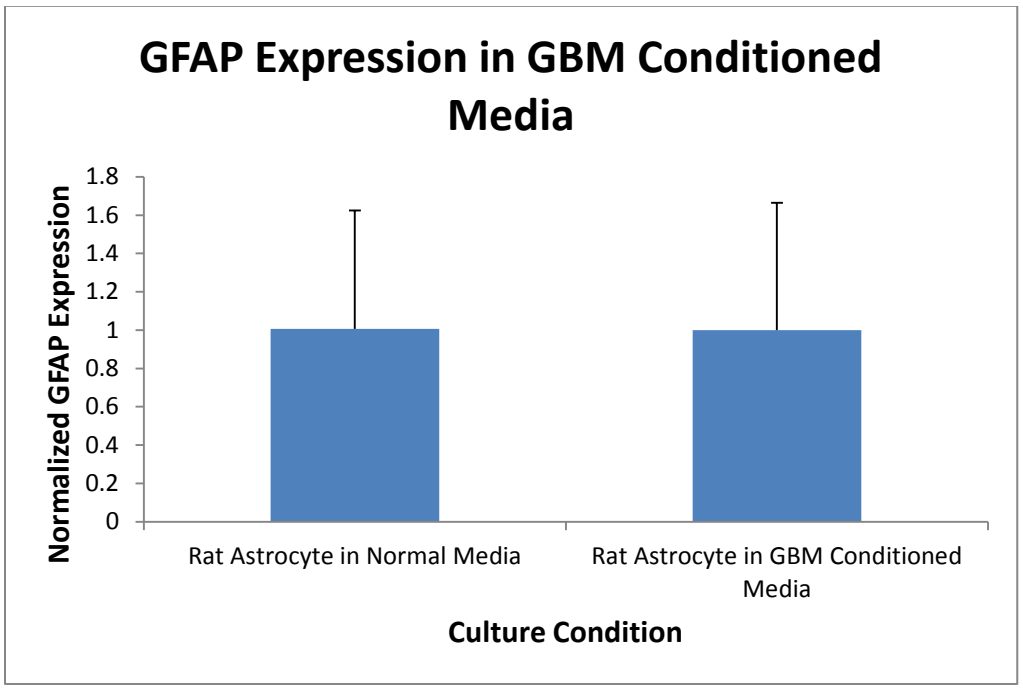

Figure 9: Astrocyte GFAP expression in GBM conditioned media versus normal conditions, n=174, no significant difference, rat astrocyte in GBM conditioned media mean is normalized to 1

3.2.3: Migration of GBM Cells in Co Culture with Rat Astrocytes

Co culture conditions of 5:1, 10:1, and 15:1 rat astrocyte:GBM cells were examined and compared to GBM cells cultured without astrocytes in rat astrocyte media electrospun fiber mats to investigate the effects that the presence of astrocytes had on GBM migration in the presence of the biomimetic topographical cues provided by the fibers. Additionally, conditioned media from rat astrocytes grown on a two dimensional tissue culture plate was added to GBM cells on fiber mats to investigate the effects of astrocyte-released stable soluble cues on GBM migration. The presence of astrocytes increased GBM migration, with a significant increase in the 10:1 and 15:1 conditions versus the control condition of GBM only in normal astrocyte media (Figure 10). Also, a significant increase in migration was seen in GBM cells cultured in astrocyte conditioned media. GBMs cultured in astrocyte conditioned media had the highest migration rate of any condition (Table 4).

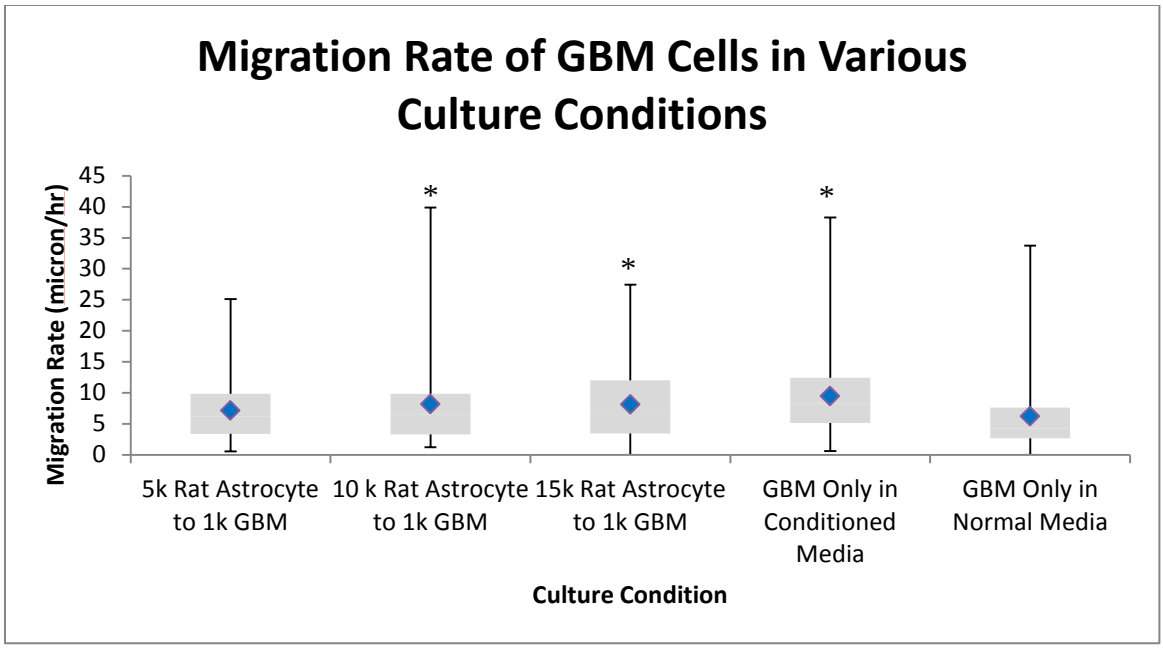

Figure 10: Migration of GBM (OSU2) cells in co culture with rat astrocytes, $p \leq 0.0379$, $n \geq 77$, * denotes significant increase compared to the control (GBM only in normal media)

Table 4: GBM migration under different culture conditions

Culture Condition	**Mean Migration Rate (micron/hr)**	**Statistically different from control?**	**p-value**
15k:1k astrocyte:GBM	8.10	Yes	0.0358
10k:1k astrocyte:GBM	8.16	Yes	0.0379
5k:1k astrocyte:GBM	7.15	No	0.2863
GBM only in conditioned media	9.45	Yes	0.0004
GBM only in unconditioned media (control)	6.18	N/A	N/A

3.2.4: GBM Migration in Media Conditioned by Astrocytes Grown on Fibers

Given the observed increase in GBM migration in the presence of media conditioned by astrocytes grown on 2D tissue culture plates, the influence of topography on production of stable soluble factors was explored by collecting conditioned media from astrocytes cultured on fibers. Ten thousand astrocytes were seeded on electrospun fibers in 100 μL of media and media was collected after either one or two days of culture. GBM cells were then cultured in this conditioned media as well as in normal, unconditioned media and migration was compared. The presence of conditioned media from fibers increased GBM migration (Figure 11), as had been seen previously using conditioned media from 2D tissue culture plates. Migration in the presence of two day conditioned media was slightly higher than in the presence of one day conditioned media, but there was not a significant difference between the two.

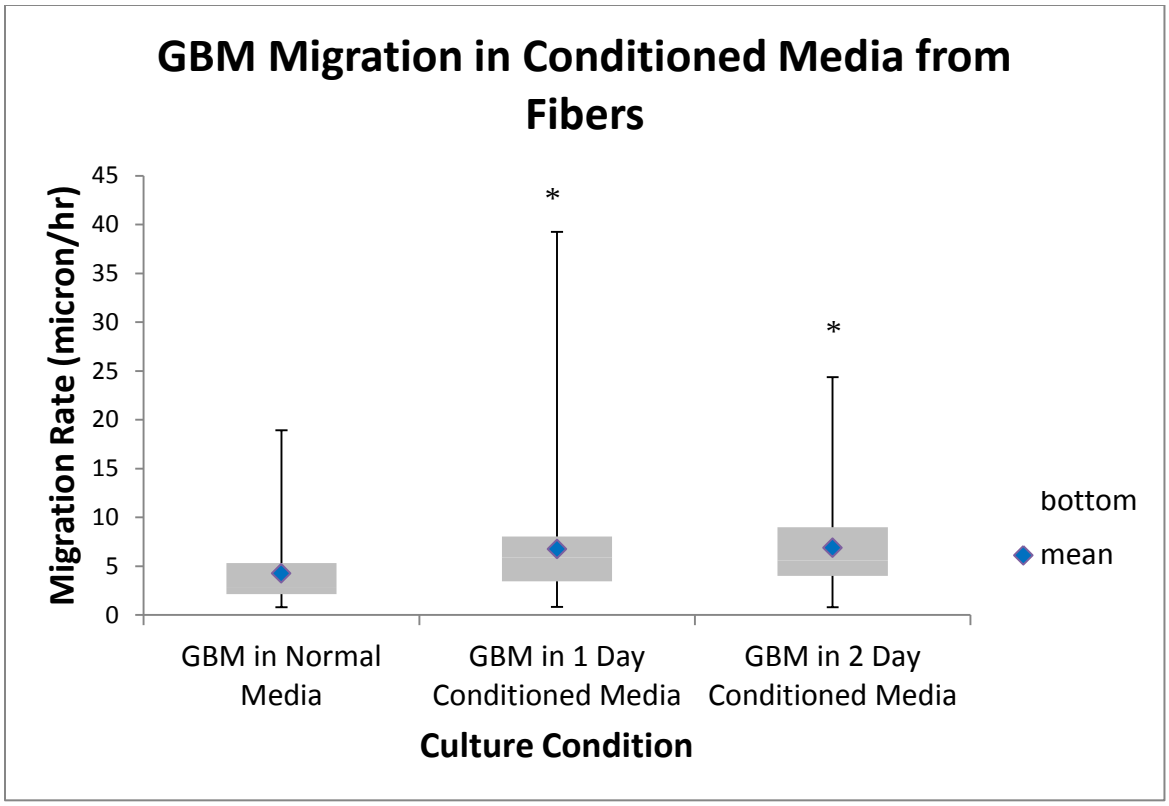

Figure 11: Migration of GBM cells in media conditioned by astrocytes grown on electrospun fiber scaffold, * denotes significant increase compared to the control (GBM in Normal Media), n≥78, p-value≤0.0003

Table 5: GBM migration in conditioned media from fibers

Culture Condition	Mean Migration Rate (micron/hr)	Statistically different from control?	p-value
GBM in 1 Day Conditioned Media	6.73847	Yes	0.0003
GBM in 2 Day Conditioned Media	6.87548	Yes	0.0003
GBM in Unconditioned Media	4.24270	N/A	N/A

3.2.5: GBM Migration in Presence of Fixed Astrocytes

To investigate the effects of the physical presence of rat astrocytes separate from other factors presented by live astrocytes on GBM migration, rat astrocytes were seeded on fibers, allowed to attach, and then fixed with paraformaldehyde. After fixation, GBM cells were seeded on the same fibers and their migration was tracked. Astrocyte concentrations of either 10000 or 15000 astrocytes were fixed to fibers. The presence of these fixed astrocytes decreased migration of GBM cells. GBMs in the presence of 15000 fixed astrocytes migrated the slowest, followed by GBMs in the presence of 10000 astrocytes, with GBMs by themselves migrating the fastest (Figure 12).

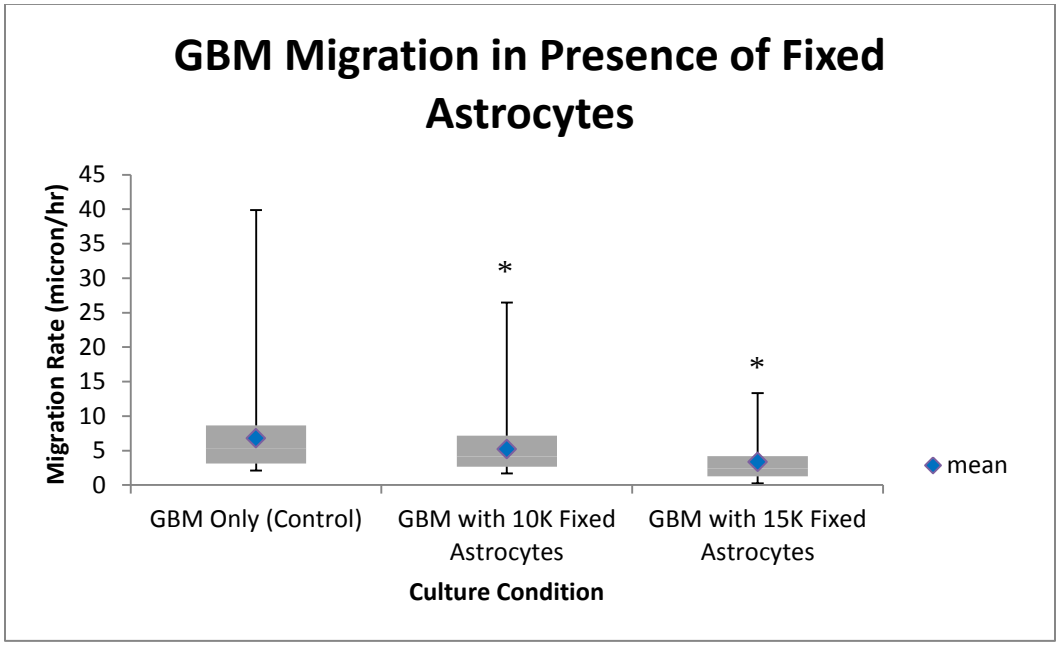

Figure 12: GBM migration in the presence of fixed astrocytes, $n \geq 102$, p-value ≤ 0.0044, * denotes significant decrease compared to control condition (GBM only)

3.2.6: GBM Migration on Astrocyte ECM Deposits

To investigate the effects of rat astrocyte deposited extracellular matrix (ECM) on GBM migration, rat astrocytes were seeded on fibers and permitted to deposit ECM for one or two days. After the proper time had elapsed, astrocytes were detached from the fibers using StemPro Accutase. ECM was clearly deposited and remained on mats following astrocyte removal (Figures 13, 14, 15) Testing of these conditions showed that deposited ECM does not influence GBM migration (Figure 16).

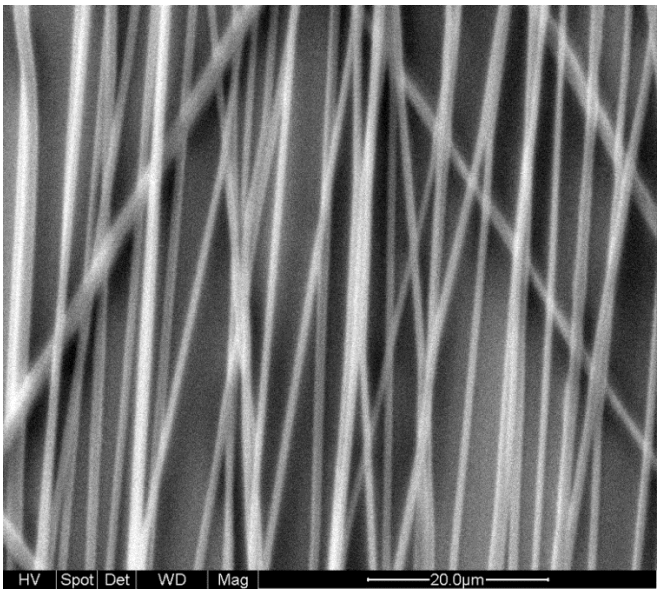

Figure 13: Scanning Electron Microscopy (SEM) image of electrospun poly (caprolactone) (PCL) fibers used in this work

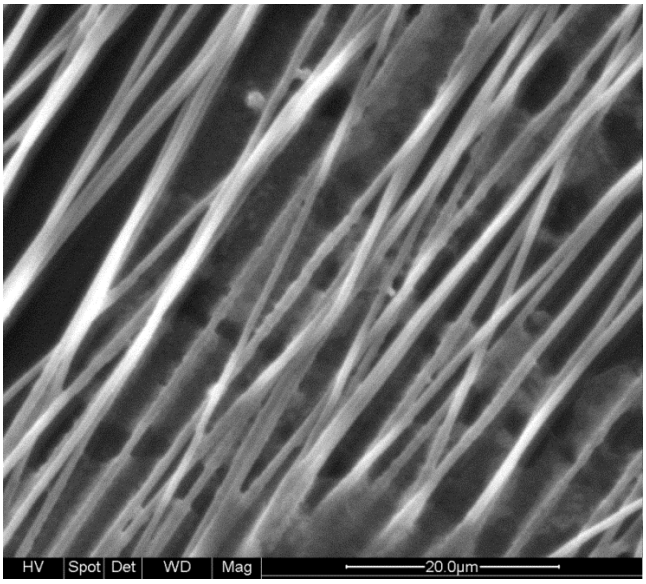

Figure 14: SEM of electrospun fibers after 1 day of astrocyte ECM deposition

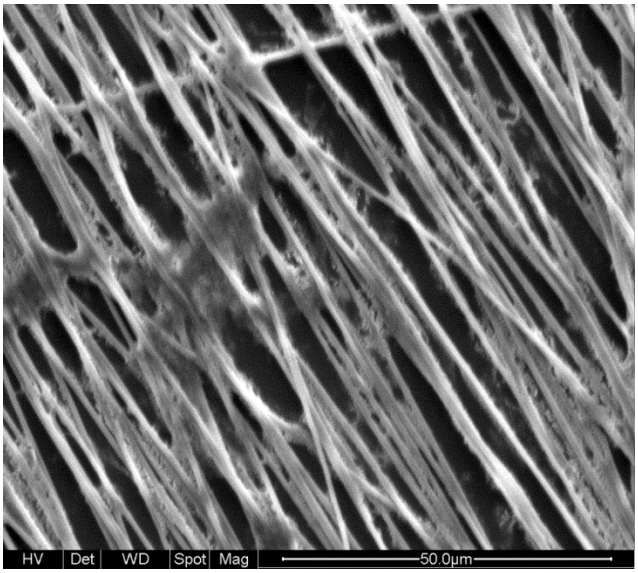

Figure 15: SEM of electrospun fibers after 2 days of astrocyte ECM deposition

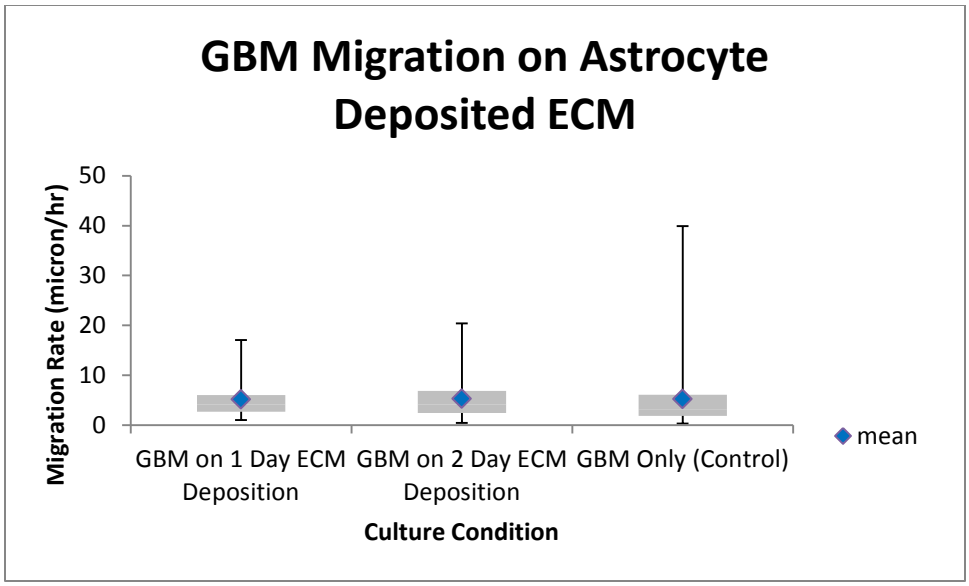

Figure 16: Migration of GBM cells in presence of ECM deposited by astrocytes for 1 or 2 days, n≥90, no significant difference seen

3.3: Discussion

3.3.1: GFAP Expression of Astrocytes

In electrospun fiber mat (EFM) based co culture systems an increase in GFAP expression was detected in astrocytes in co culture versus astrocytes cultured alone. This GFAP up regulation increased with increasing GBM cells. This suggests that the astrocytes are able to sense the presence of the GBM cells and become reactive in response. This response was not unexpected as similar results have been seen in previous co culture studies in 2D.[18 79 82] However, to the best of our knowledge this is the first study to evaluate astrocyte-GBM co culture an EFM environment that mimics topographical features of native tumor migration in the brain. For this reason it was of interest to see if astrocyte behavior remained the same in this environment compared

to others. A number of factors were identified as being possible causes of the observed GFAP increase, including the presence of surface markers on GBM cells, the presence of proteins in GBM deposited ECM, and soluble factors released into the media by GBM cells. To evaluate the possibility of a stable soluble factor influence, astrocyte culture was performed in GBM conditioned media. No difference was seen in GFAP expression between astrocytes in normal media versus GBM conditioned media. This data suggests that GBM released stable soluble factors do not contribute to the observed astrocyte response. This was not entirely unexpected because previous work had shown that conditioned media from glioma cells had inconsistent effects on normal brain cell aggregate viability, with conditioned media from one cell line showing no obvious effect on normal cell viability,[76] suggesting that glioma soluble factors may not have a major influence on surrounding normal brain. Other possible signals include the presence of GBM deposited ECM, the physical presence of GBM cells, or gap junctions formed between astrocytes and GBM cells. These possibilities should be more thoroughly investigated in future studies.

3.3.2: Migration of GBM Cells in Co Culture

GBM cells in co culture with rat astrocytes showed an increase in migration versus GBM cells cultured alone (control condition). Significant differences were seen between migration of GBMs in the control condition and GBMs co cultured in both 10:1 and 15:1 rat astrocyte:GBM ratios. A previous study had seen a decrease in glioma

migration when astrocyte-glioma gap junctions were inhibited,[79] suggesting that interactions between the two cell types positively influence migration of glioma cells, similar to our experimental observations. Additionally, previous work showed that aggregates of astrocytes do not repel invading glioma cells whereas aggregates of glioma cells did repel invading gliomas,[83] suggesting that glioma invasion and migration may be promoted in the presence of astrocytes. However, whereas the presence of astrocytes certainly increased GBM migration, this increase did not correlate with increasing numbers of astrocytes. Migration was the highest in the 10:1 astrocyte:GBM ratio and slightly lower in the 15:1 astrocyte:GBM ratio, with the 5:1 ratio seeing the least migration of the three. This suggests that multiple factors may be playing a role in promoting or inhibiting migration. Potential factors identified and investigated included soluble cues released from astrocytes, the physical presence of astrocytes, and astrocyte deposited ECM on the fibers.

The effects of astrocyte released soluble cues were first investigated by preparing conditioned media from astrocytes grown on a two dimensional culture plate, culturing GBM cells in this conditioned media, and comparing migration of these cells to migration of GBM cells in unconditioned media. This conditioned media significantly increased migration. A shortcoming of using this conditioned media, however, was that it was conditioned by astrocytes being grown on a tissue culture dish instead of on electrospun fibers as in the co culture experiments. Additionally, there were many more astrocytes per milliliter of media on the tissue culture plates than in the co culture

studies. A second experiment was performed in which astrocytes were grown on electrospun fibers at a density of 10000 cells per well and conditioned media from these astrocytes was used to more closely mimic the environment from the co culture studies. It was seen that the presence of astrocyte conditioned media from fibers caused a significant increase in GBM migration, as had been seen with astrocyte conditioned media from 2D tissue culture plates. This data would suggest that rat astrocytes release stable soluble cues that interact with GBM cells and cause an up regulation in a cellular migration pathway. Previous work has shown that microglia cells release factors increasing glioma migration in a Boyden chamber assay,[84] and our results further demonstrate that other cell types might also play an important role in increasing cell migration. However, the presence of soluble factors does not appear to be the only factor effecting GBM migration in presence of rat astrocytes. If this were the case, it would be expected that as the number of rat astrocytes in co culture with GBM cells increases, the migration of the GBM cells should also increase. Our data did not show this trend, however, as no difference in GBM migration was observed between the 10:1 astrocyte:GBM and 15:1 conditions. Thus, it can be concluded that there are other components that effect migration as well. It was hypothesized that migration in co-culture is a function of soluble factors, ECM cues, physical presence of cells, and gap junction interactions between living cells. It was hypothesized that some of these factors act to increase migration, some act to decrease migration, and some may have no effect at all.

The effects of the physical presence of astrocytes, namely the presence of astrocyte surface markers influencing migration or the presence of the astrocytes acting as a barrier, were investigated by fixing astrocytes to fibers and tracking GBM migration in this environment. GBM migration decreased in the presence of fixed astrocytes, suggesting that the physical presence of astrocytes acts as a barrier to GBM movement and migration. This migration decrease was directly proportional to increasing numbers of fixed astrocytes. This seems to be part of the answer as to why increases in GBM migration in our co culture system were not directly proportional to increasing astrocyte number. Whereas the presence of more astrocytes leads to release of more stable soluble factors, which promote migration, it also leads to more barriers to GBM movement or more migration inhibiting astrocyte surface markers, providing a countermeasure to the migration increase caused by soluble factors.

The effects of the presence of astrocyte deposited ECM on fibers were investigated by growing astrocytes on fibers for 1-2 days and removing the astrocytes while leaving deposited ECM intact. The presence of ECM did not have any effect on GBM migration. This was surprising as some effect was expected to be seen, whether it be a reduction in migration rate due to the fact that GBM cells must break down ECM *in vivo* before they can migrate[46] or an increase in migration due to stimulation by presence of certain ECM components.[85] The lack of reduction in migration may be due to the fact that ECM was deposited on the fibers before seeding GBM cells, thus not allowing GBM cells to be surrounded by ECM as they would be *in vivo*.

Chapter 4: Conclusions and Future Work

4.1: Conclusions

This work examines the interactions between astrocytes and GBM cells and was done in a more physiologically relevant system meant to closely mimic the environment seen by migrating GBM cells *in vivo*, specifically the periphery of blood vessels and white matter tracts in the brain. Additionally, it used a new patient cell line of GBMs that is less likely to be mutated from its *in vivo* predecessor than cell lines that have been maintained for decades.

Astrocytes became reactive in the presence of GBM cells, similar to what has been seen in previous co culture systems.[18 79 80] The mechanism by which these cells became reactive was not elucidated in this study, however. It was originally hypothesized that astrocytes sensed the presence of the foreign GBM cells through GBM released soluble factors, leading to an increase in GFAP expression. The results of our experiments studying GFAP expression of astrocytes in GBM conditioned media did not show any difference in GFAP expression between these astrocytes and astrocytes cultured under normal conditions. These results do not therefore support the hypothesis that GBM stable soluble factors are responsible for astrocytereactivity.

Other possible mechanisms, such as presence of GBM deposited ECM and GBM-astrocyte gap junctions, are potential avenues to investigate in the future.

GBM cells increased migration rates when co cultured with rat astrocytes, which was a very interesting and surprising discovery. Several different factors affected GBM migration in the presence of astrocytes. The presence of astrocyte released stable soluble factors in astrocyte conditioned medium promoted GBM migration. Also, the physical presence of astrocytes seemed to act as a barrier to GBM migration, leading to a decrease in migration rate. Taken together, there are several factors contributing different effects on GBM migration in presence of astrocytes. By identifying factors that affect GBM migration and their mechanism, we can gain a better understanding of the mechanisms of GBM migration and thus develop better ways to prevent it.

4.2: Future Work

One of the major shortcomings of this work is that fact that it involves co culturing human GBM cells with rat astrocytes. Whereas this is a valuable study that can produce many valuable insights, it is important to have a model that is as physiologically relevant as possible. Thus, to more closely mimic the human brain environment *in vivo*, it would be advantageous to co culture GBM cells with human astrocytes and verify the results observed in this work. This would definitely be an important and interesting path to investigate in the future. It would also be interesting to further investigate the factors affecting the GFAP up regulation in rat astrocytes seen

in the co culture model presented in this report. The presence of GBM stable soluble factors was investigated, but there are many other factors that may be playing a role. Because GBM conditioned media was collected and transferred to rat astrocyte culture, it is possible that unstable, fast degrading soluble factors that may have been making a difference in culture were degraded and thus were not accounted for. This possibility should be explored using a transwell system. The presence of ECM deposited by GBM cells as a possible cue for GFAP up regulation should also be explored as a possibility. Additionally investigating the effects of gap junctions formed between the astrocytes and GBM cells could shed more light on the mechanism for astrocyte recognition of GBM presence.

It would also be of interest to further investigate the factors causing increased GBM migration in the presence of astrocytes. The contributions of stable soluble cues, deposited ECM, and physical presence of cells have been investigated in this work, but the effects of gap junctions between astrocytes and GBM cells were not investigated. It is highly possible that these gap junctions play a major role in GBM migration, so investigation of gap junction effects would be an investigation of great interest in the future. Another factor that may have contributed to the migration differences seen in co culture is the fact that more cells were present in co culture systems than in the control, thus depleting nutrients in the media much further and faster than in the control condition. This would be a very interesting factor to investigate. It would also be relevant to investigate the effects of unstable, fast degrading soluble factors being

released by astrocytes. This could be investigated by using a transwell system, seeding astrocytes in the top chamber and GBM cells in the bottom and using a filter with small enough pore size to prevent astrocyte migration to the bottom but not prevent soluble factor transport. Finally, after having seen a major increase in GBM migration in the presence of astrocyte released stable soluble cues, it would be of great interest to identify the specific cues promoting this migration and the biological pathways in which they participate. As more becomes known and understood about GBM behavior and the factors causing and influencing it, we can develop more effective ways to treat GBM tumors and combat GBM migration and the growth of secondary tumors.

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Appendix A: Standard Operating Procedures

Biological Waste Disposal

Gang Ruan

Rev 2

July 21, 2007

Purpose

Disposal of biological waste materials from the working area without causing threats to the environment.

Materials

Waste to be disposed

Autoclave

Bleach

Biohazard plastic bag/boxes

Hard plastic sharps container (designated biohazard waste)

Safety Concerns and PPE:

Biohazard waste, by definition, may pose a biological threat to the creator. Waste should be disposed of according to the following guidelines to minimize that threat. Solid waste including paper towels, pipettes, pipet tips, and culture dishes may be treated with either autoclaving or bleaching. See procedure below. Sharps waste including needles and broken glass should be deposited directly in a biohazard sharps container for disposal. Liquid waste including discarded medium, serum, and saline should be treated with bleach before disposal. Please note the following additional hazards.

- The high temperature/high pressure in autoclave may be a threat to the operator's safety. Please read autoclave SOP before use.
- Bleach presents chemical hazard. Use gloves, lab coat and glasses to prevent splash/spills.
- Sharps can present a cut hazard. Please handle with care. Keep needles covered when not in use, and dispose of materials promptly.
- Operator may need to use a sterile culture hood. Please read the SOP on the sterile culture hood before proceeding.

PPE Recommendations: Operators should wear lab coat, safety glasses, and nitrile or latex gloves. Additionally, operators may perform portions of work in a sterile culture hood.

Procedure

1. Biohazard waste is waste which has been exposed to cells, tissue, and reagents that contain biohazardous agents (i.e., drugs, neurotoxins, etc.).
2. All contaminated materials must be sterilized before disposal. Waste can be divided into three basic categories: **liquid waste, solid waste, and sharps waste**.
3. **Liquid waste** consists of biological medium, cell culture solutions, and liquids with biological activity. Liquid solutions should be treated with bleach (usually 1:10 to 1:1 concentration bleach:waste) before disposal. Solutions that do not pose a chemical threat to the individual after treatment (i.e., salt solutions and buffers, cell culture medium) can be disposed of in the sink. Solutions that do pose a threat to the individual (i.e., formaldehyde, reagents containing active quantities of drugs) should be disposed of as separate liquid waste and tagged accordingly. Solutions that are particularly dangerous (i.e., neurotoxins, formaldehyde) should be disposed of immediately. Contact EHS to schedule for the pickup (through phone 614-292-1284 or internet www.ehs.ohio-state.edu).

4. **Solid waste** includes disposable pipettes, culture dishes, pipette tips, kim wipes etc. Solid waste that has been exposed to liquid solutions (i.e., cell culture dishes, pipettes) must be treated before disposal. These solids may be treated either through bleach exposure (see above) or by autoclaving (see Autoclave SOP for procedures to treat waste through this method). Kimwipes and paper towels do not need to be treated in this method, as it is unlikely they harbor significant quantities of liquid. After treatment, waste should be placed in red biohazard plastic bag/boxes for collection. When the box is full, contact EHS to schedule for the pickup (through phone 614-292-1284 or internet www.ehs.ohio-state.edu).
5. **Sharps waste** includes needles, broken glass, and other sharp objects that may have come into contact with biohazards. All sharp items must be placed in a designated hard plastic sharps container (designated biohazard waste). These containers contain plastic closures that should be sealed when not in use. When containers are full contact EHS to schedule for the pickup (through phone 614-292-1284 or internet www.ehs.ohio-state.edu).
6. **For more information, check the website of EHS.**

Counting Cells

Jessica Winter

Rev 2

07/13/07

Purpose: Cells can be counted to establish the growth rate of the culture, to allow for experimental plating at a known density, or to determine the proper density for cell freezing. Cell counting is performed using a sample of the population, and then statistically extrapolating that value to determine the concentration of the total population. Cells are counted on a grid, called a hemocytometer, which is a chamber that holds a fixed volume.

Materials:

Phosphate buffered saline (PBS) without Calcium and Magnesium [Sigma P3813]

Trypsin-EDTA (for highly adherent cells only) [Sigma T4049]

Cell Culture Medium

15 ml sterile conical tube

Sterile pipettes (5 and 10 ml)

200 uL Pipetman

20 uL Pipetman

Sterile tips for 20 and 200 uL Pipetman

Microcentrifuge tube

Microcentrifuge tube rack

Kimwipes

EtOH

Hemacytometer

Counting Aid

Calculator

Safety Concerns and PPE:

Cells must be counted in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device. In addition, counting cells exposes the user to biohazards. Trypsin/EDTA (TRED) is a protease meaning it dissolves proteins. Operators should wear gloves to protect against this hazard.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When counting all operators should wear nitrile or latex gloves, lab coat, and safety glasses.

Procedure:[1]

1. EtOH wash hemacytometer and cover using Kimwipes. Place next to microscope, along with 10 uL Pipetman, tips, counting aid, and microfuge tube rack. Hemacytometer must be completely dry to prevent cell streaming!
2. EtOH wash required pipettes, medium, TRED, PBS, and conical and place in hood.
3. In sterile culture hood, aspirate medium using sterile pasteur pipette.
4. Add 3 ml PBS,[2,3] swirl dish to coat, and aspirate with sterile pasteur pipette.

5. *If cells are highly adherent (e.g., fibroblast) only*, add 2 ml trypsin-EDTA (TRED)[4]. Observe in microscope for 2-3 minutes, until cells exhibit a rounded morphology and begin to detach from dish. If possible, aspirate TRED.[5]

[1] Steps 2-9 steps are same as procedure for passaging cells.
[2] PBS without Ca and Mg is added to rinse away remaining Ca ions and debris. Ca is important in cell adhesion, and its removal will aide isolation of the cells.
[3] 3 ml for 10 cm dish. Otherwise use about 1/3 the volume used for feeding.

6. Add 8 ml culture medium,[6] and starting at one corner of the dish, wash the surface of the dish from the middle toward the outside. Rotate the dish until all surfaces have been washed.
7. Aspirate medium and cells into a sterile 15 ml conical tube.
8. Centrifuge at 3000-4000 RPM for 3 minutes at 25 ºC.
9. Without disturbing cell pellet, carefully aspirate as much media as possible.
10. Resuspend cells in medium to produce desired cell density.[7]
11. EtOH wash microfuge tube and place in tube rack in hood.
12. EtOH wash 200 uL Pipetman dialed to 50 uL and place on top of tips box in hood.
13. Opening tips box from the front, and carefully place Pipetman in box and attach tip. Be careful to place hands over tip box. Place Pipetman, with tip, on box with tip to rear of hood.
14. Remove capped 150 ml conical from hood and vortex for a few seconds. Then, quickly return to hood and remove 50 uL of cell solution using 200 uL Pipetman. Place 50 uL in microfuge tube.
15. Remove microfuge tube from hood and place in tube rack next to microscope.
16. Center the coverslip, set 20 uL pipette to 10 uL, attach tip.
17. Briefly vortex cell suspension, and place 10 uL in each side of the hemacytometer. [See figure for tip placement.

18. Place hemacytometer in the microscope using phase illumination and 10X objective.
19. The hemacytometer [see Figure below] contains a grid of multiple squares. The nine large squares have lengths of 1 mm/side. The corner 1mm squares are divided into a series of smaller 16 smaller squares, each with a side of 250 um. The central square is divided into 25 smaller squares, each with a side of 200 um. Each of these smaller squares is further divided into 4 smallest squares, each with a side of 50 um.
20. Locate the central square and briefly assess cell density. For best results, a cell density between 20-50 cells/square is required.[8,9]

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[4] Trypsin is a protease that digests cell connections to the dish. EDTA is a chelator that will bind remaining Ca ions, inhibiting cell attachment.

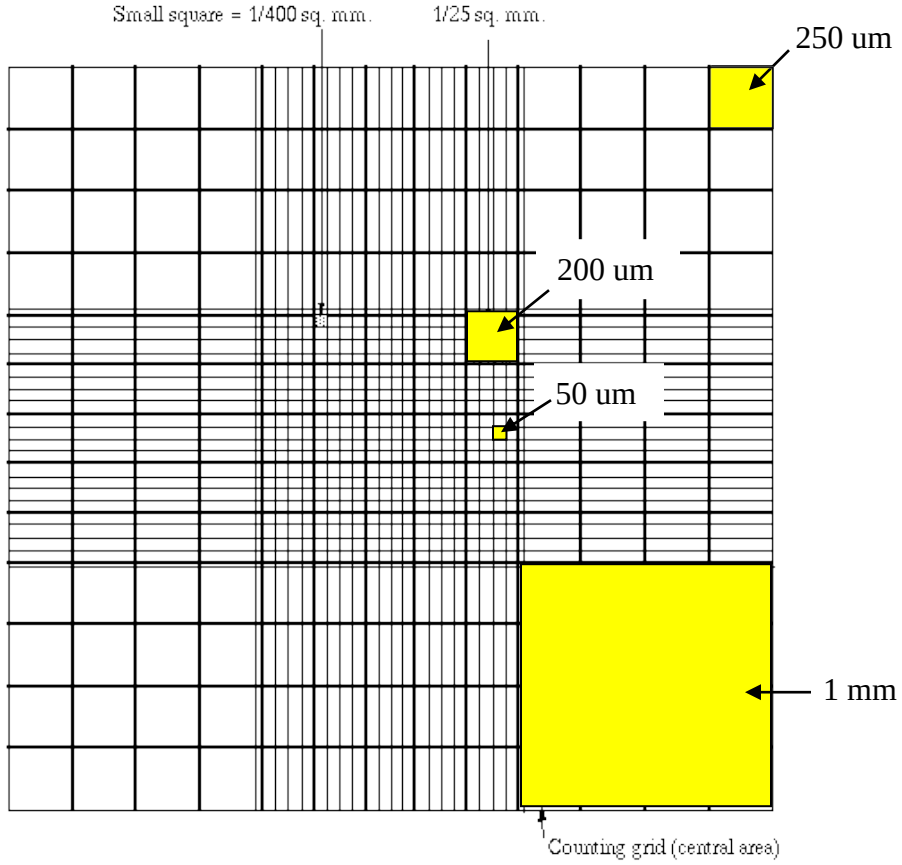
[5] It used to be standard to rap the sides of the dish to detach cells at this stage. This is now viewed negatively as it can cause significant cell aggregation.

[6] For 10 cm dish, else 80% of feeding volume.

[7] It can be difficult to gauge the medium needed, when the concentration is not known. As a guideline a confluent dish will contain 1-2 million cells per dish (for 10 cm dish). Based on the estimated percent confluency an approximate concentration can be determined, and this can be used to estimate how much medium to add. If the estimate is off, the pellet can always be centrifuged and resuspended in the correct amount of medium. In general, it is better to be too concentrated (easy to dilute if needed) than too sparse.

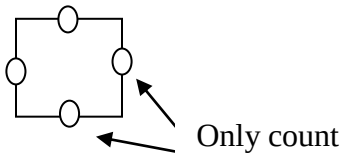
[8] For best statistical accuracy cell counts should be between 20-50 cells/square. If cells are more concentrated, aliquot can be dilutes with medium. If diluted, the final concentration must be adjusted for this addition. If cells

21. If cell density is not correct, it can be adjusted by diluting with fresh medium. (Do not return pipette to medium bottle after placing in microfuge tube to prevent compromising sterility.) Additionally, sample can be centrifuged and resuspended in a smaller amount of medium.



Hemacytometer grid. The numbers given are the side length of each square shown in yellow.

22. If cell density is correct, cells are ready to be counted. For a quick count, count the cells in each of the four corner squares and the central square on both sides of the hemacytometer. For a more accurate assessment, count the cells in each of the nine squares on both sides. The counting aid can be used to keep track of cell square counts. To avoid double counting, cells that fall on the border of the square should only be counted on the right and lower, lines.



23. The concentrations of each square should then be averaged together to obtain an average number of cells/ 1 mm square.

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are two few, sample can be centrifuged again and resuspended in a smaller volume of medium. If this is not possible, then smaller concentrations can be evaluated, but will not necessarily be accurate.

[9] If more than 10% of cells are present in clumps, sample must be triturated to break-up clumps before evaluating.

24. Each square contains, with cover-slip in place, a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:

$$\textbf{Cells Per ml} = \frac{\text{Average Cells}}{\text{Square}} * \frac{\text{square}}{10^{-4}\ \text{cm}^3} * \frac{1\ \text{cm}^3}{1\,\text{ml}} * \text{dilution factor} = \frac{\text{Cells x}10^4}{\text{ml}}$$

Ex: If the average count per square is 45 cells and the dilution factor is 5. Then the number of cells = 45 * 5 * 10^4 = 2.25 x 10^6 cells/ml.

Feeding Cells

Jessica Winter

07/13/2007

Rev. 2

Purpose: This procedure describes the method for feeding adherent cells. Cells should generally be fed every 2-3 days to prevent waste build-up in the medium and to replenish nutrients. This is regardless of the color of the medium.

Materials:

Sterile pipette

Supplemented Culture Medium

Sterile Pasteur pipette

Safety Concerns and PPE:

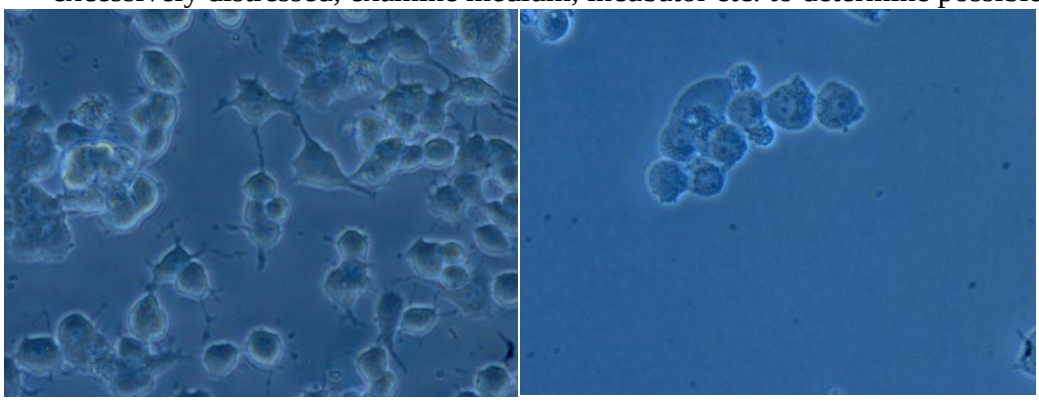
Cells must be fed in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When feeding all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure:

1. Place supplemented medium in 37 ºC water bath to warm for ~ 15 minutes.
2. Remove cells to be fed from cell culture incubator.[10]
3. Examine cells on microscope to ensure proper morphology, or cell shape. If cells appear excessively distressed, examine medium, incubator etc. to determine possible cause.



Live PC12 Cells (20X) *Dead PC12 Cells*[11]

4. Open sterile culture hood and turn on blower and lights.
5. Wipe base of hood with 70% ethanol from back to front of hood. (This prevents overreaching of areas already sterilized with EtOH)
6. Wipe down warmed medium and pipette; place in sterile culture hood.
7. Place cell culture dish in sterile culture hood.
8. Turn on vacuum.[12]
9. Turn Pasteur pipette box on side and remove lid. Place lid under box to slightly elevate.[13]
10. Remove sterile Pasteur pipette from box[14] and attach to end of vacuum line, while maintaining pipette tip near the top of the hood (most sterile part). Do not touch pipette

[10] Handle cell culture dishes with care. If dishes open for even a second devastating bacterial or fungal contamination can occur. This is especially important for smaller diameter cell culture dishes, which are more difficult to handle. I usually firmly grip the dish at both the top and bottom using my thumb (top of dish) and fingers (bottom of dish). If dish comes open, cells should be discarded, as they can contaminate the rest of the incubator in the case of bacterial or fungal contamination.

[2] Dead cells can be indicated by many things: cloudy medium, a rounded morphology, a more visible nucleus, blebbing (dark bulges of the cell membrane), excessive vesicles, excessive floating debris, and extruded cytoplasm.

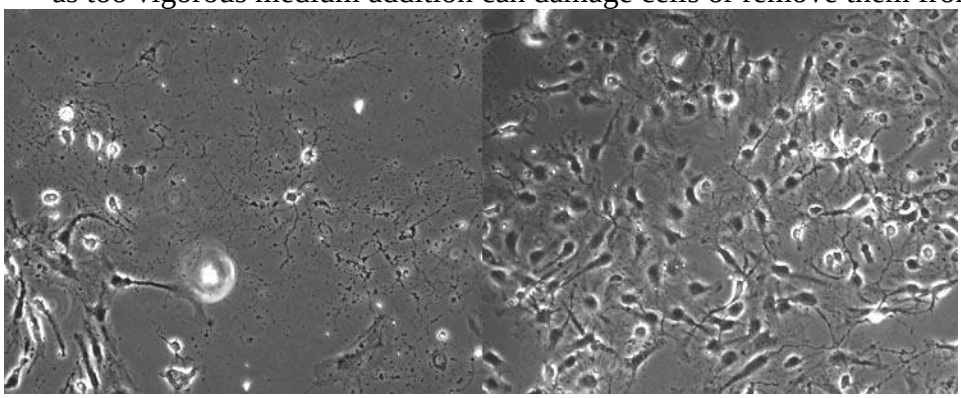
[12] The vacuum aspirator bottle contains large amounts of bleach to sterilize aspirated medium. Because of this we only turn it on when in use to minimize the smell.

[13] Be careful to not reach over the top of pipette box. If this occurs box must be re-autoclaved.

[14] Box remains in hood.

tip to anything. *If any pipette tip ever touches a non-sterile object or the exterior of a sterile object it must be discarded.*

11. Open the cell culture dish using the clam shell technique. Using your left hand, grip the lid with your thumb on the base of the lid (towards you) and your forefinger on the top of the lid (away from you). Place your middle finger on the top of the lower dish (not the lid). Maintaining the position of your middle finger and thumb, move your forefinger towards you to open the dish slightly.
12. Aspirate cell culture medium by placing Pasteur pipette in the far left side of the dish.
13. When complete, close lid.
14. Place used Pasteur pipette in red biohazard bag at back of hood.
15. Open/crack lid of sterile culture medium. Replace lid gently on top of bottle.
16. Holding pipette in right hand, attach sterile pipette to pipette-aid.
17. Remove lid from sterile culture medium using left hand, and hold facing the side of the bottle.
18. Using right hand, remove necessary amount of cell culture medium from pre-warmed supplemented cell culture medium bottle. (For 10 cm dish, 10 ml, For 6 cm dish, 5 ml, For 3 cm dish, 2 ml, For 24 well plate 500-600 ul, For 96 well plate, 200-300 ul).
19. Using left hand replace lid on sterile culture medium bottle. Keep pipette elevated and not touching anything with right hand.
20. Using left hand, employ clamshell technique to crack open the culture dish.
21. Slowly add cell culture medium to far left side of dish. Be careful to add medium slowly as too vigorous medium addition can damage cells or remove them from the dish.[15]



Rat Neonatal Cortical Cells (RNC) fed too vigorously (left) and fed appropriately (right).

22. Close lid of culture dish.
23. Remove pipette from pipette aid and place in red biohazard bag at back of culture hood.
24. Remove cell culture dish and place in incubator.
25. Close cell culture medium and return to refrigerator.[16]
26. Remove pipette aid and other elements from hood.
27. Close Pasteur pipette box and place upright in hood.

[15] I do not depress the pipette aid all the way. I usually use gently pressure to slowly add the medium.
[16] Culture medium is light sensitive. Best to place at the rear of refrigerator or in a dark plastic bag.

28. Wipe down hood with 70% ethanol.
29. Close hood and turn off lights and blower.

Freezing Cells[17]

Jessica Winter

Rev 2.

07/13/2007

Purpose: Cells are frozen to preserve a certain passage number for later use and when they are not needed for some time. Cells can persist in a frozen state for some time, at least a year, and often several years.

Materials [excluding those needed for counting cells]:

DMSO (dimethylsulfoxide), sterile, cell culture tested

Cryovials

Styrofoam containers (e.g., 15 conical holders)

Paper towels

Aluminum foil

Lab tape

Safety Concerns and PPE:

Cells must be frozen in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device. In addition, counting cells exposes the

[17] Follow procedure for counting cells first.

user to biohazards. Trypsin/EDTA (TRED) is a protease meaning it dissolves proteins. Operators should wear gloves to protect against this hazard. Operators are also exposed to cryohazards when freezing cells. Please read the SOP on dealing with cryoagents before proceeding.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.
- When freezing, operators should avoid splashing of cryogenic fluids.

PPE: When preparing samples all operators should wear latex or nitrile gloves, lab coat, and safety glasses. To freeze samples operators should use cyrogloves, lab coat, and safety glasses.

Procedure:

1. In addition to materials needed for passaging and counting, EtOH wash DMSO and cryovials and place in sterile culture hood.
2. Follow the procedure for counting cells. Briefly, cells should be passaged into 15 ml conicals, aliquots taken, and the density determined. Target cell density is 1 x 10^6 cells/ml. Because confluent 10 cm dishes usually contain between 1-2 x 10^6 cells only 1-2 ml may be needed for resuspension.
3. Based on measured cell concentrations, adjust cell density to achieve 1 x 10^6 cells/ml. This can be accomplished by adding medium to dilute, or by centrifuging pellet and resuspending a smaller amount of medium. A minimum volume of 1 ml is required.[18]
4. Place 50 ul of DMSO/ml cells in conical.
5. Aliquot cells in 1 ml increments into cryovials in sterile culture hood.
6. Label aliquots with cell concentration, passage number, initials and date.
7. Remove cryovials from hood and place in Styrofoam container.
8. Wrap container with paper towels to cover. I usually use 4 open towels, two for each direction, secured with lab tape.
9. Wrap with aluminum foil to cover.
10. Place in -80 ºC freezer.
11. Next day, remove from Styrofoam and place in long term microfuge tube storage boxes.

[18] If cells are not sufficiently numerous to allow for 1 ml of cells at 1 x 106 cells/ml, place cells in 1 ml of medium at a reduced concentration. This may affect cell viability at thawing.

GFAP Staining[19,]

John Larison , Alex Hissong
Rev 2
10/25/2011

Purpose: To use immunofluorescent staining to mark the presence of the intracellular protein GFAP in monolayer culture.

Materials:

PBS (Sigma P4417)
Sucrose (Sigma S0389)
1M NaOH (Sigma-Aldrich 480878)
Paraformaldehyde (Sigma-Aldrich 158127)
Triton X-100 (Sigma T8787)
BSA (Jackson ImmunoResearch 001-000-161)
Anti-Integrin α3β1, clone M-KID2 (Millipore MAB1992)
FITC-Goat Anti-Mouse 2º Ab (Jackson 115-095-166)
Chemical fume hood
Sterile tissue culture hood
Fluorescence microscope
Incubator
Refrigerator
Rocker
Hot/Stir plate
Stir bar
Analytical balance
Microspatula
Weighing paper
10-mL beaker
Micropipettes
Micropipette tips
Microfuge tubes
Large beaker
70% Ethanol

Safety Concerns and PPE:

Paraformaldehyde is a toxin, teratogen, and suspected carcinogen. It is also an irritant to the skin, respiratory system, and sensory systems. Use extreme caution while handling paraformaldehyde. The powder has the potential to become airborne.

- Wear safety goggles and a face shield to prevent contact with the eyes.
- Wear a respirator to avoid inhalation.
- Wear gloves at all times. Wearing two gloves on each hand is recommended.
- Only work with paraformaldehyde in a chemical fume hood.
- It may be helpful to line the bench with paper towels that can be easily cleaned up in the event of a spill.
- Clean all contacting surfaces with 70% ethanol, including gloves, sleeves, etc…

D-PBS is a saline solution, and therefore an eye irritant.

- Wear safety glasses and exercise caution when handling D-PBS to avoid contact with eyes.

The stains must only be opened in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.

[19] This SOP requires the use of a chemical fume hood, a sterile tissue culture hood, and a fluorescence microscope. Working knowledge of all three is assumed for the purposes of these instructions. Refer to the respective SOPs for further help or ask for assistance.

- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

UV light can be harmful to the eyes.

- Wear UV-resistant safety glasses while imaging the cells.

PPE: When immunostaining all operators should wear latex or nitrile gloves, lab coat, and safety glasses. Face shield and respirator should be worn when handling paraformaldehyde. Butyl rubber gloves may also be worn when handling paraformaldehyde for extra protection.

When imaging cells all operators should wear latex or nitrile gloves, lab coat, and UV-protective safety glasses.

Figure out how much of each solution you will need for fixing and staining. I use 400 uL or 200 uL fixing, triton and blocking solution per well for 24 and 96 well plates respectively. These solutions are cheap and easily made. I use 100 uL and 50 uL per well for 24 and 96 well plates respectively when applying the staining solutions. For staining solutions it is necessary to cover the staining surface and maintain a wet surface during all incubation times but remember the antibodies are expensive and an excess of solution is not necessary. Scale solution formulas as appropriate. Remember the paraformaldehyde solution is heated so you will lose a little to evaporation.

Procedure (Preparing Fixing Solution):

1. Heat 2 mL of PBS in a 10 mL beaker to 60°C on a hot plate. It is not important that the solution is exactly 60°C, only that it is heated. Stir with a very small stir bar.
2. While the solution is heating, add 80 mg sucrose (40 mg/mL) and 20 µL of 1M NaOH (10 µL/mL).
3. Once the sucrose has dissolved and the solution has been warmed to approximately 60°C, add 80 mg of paraformaldehyde (40 mg/mL).
4. Before fixing the cells with this solution, allow the solution to cool to room temperature after the paraformaldehyde has dissolved.

Procedure (Preparing Triton Solution):

1. Add 500 µL of PBS to a microfuge tube.
2. Add 1 µL of Triton (2 µL/mL) to the PBS. Mix the two liquids by pumping with the micropipette several times.

Procedure (Preparing Blocking Solution):

1. Add 500 µL of PBS to a microfuge tube.
2. Add 15 mg of BSA (30 mg/mL) to the PBS.

Procedure (Preparing Stains)[20]:

1. Add 500 µL of PBS to a microfuge tube.
2. Add 15 mg of BSA (30 mg/mL) to the PBS. (to prevent non-specific binding)
3. Add 2.5 µL of mouse anti-human integrin GFAP primary stain to the tube[21].
4. Add 500 µL of PBS to another microfuge tube.
5. Add 15 mg of BSA (30 mg/mL) to the PBS.
6. Add 1 µL of goat anti-mouse secondary stain stock to that tube[21].

Procedure (Staining):

1. Wash the cells with warm PBS by adding ~200 µL to each well and swirling. Aspirate the spent PBS. Be gentle while adding or removing solutions from the same position each time.
2. Fix the cells with 400 µL of the paraformaldehyde solution for 20 minutes at room temperature. After paraformaldehyde is added further work must done in the hood until day 2. After 20 minutes, aspirate the spent fixing solution as with the PBS wash in step 1. The paraformaldehyde presents a strong biological hazard. It, along with all other solutions that subsequently contact the gels, must be disposed of as biohazardous waste. While completing the stain procedure, dispose of all hazardous solutions in a beaker of ethanol to be kept in the hood. When the procedure is over, all laboratory supplies that contacted paraformaldehyde must be doused with ethanol, and then cleaned as usual.
3. Wash with PBS.
4. Extract with 400 µL of the Triton solution for 15 minutes at room temperature.
5. Wash with PBS.
6. Add 400 µL of the blocking solution to each well and incubate for 15 minutes.
7. Add 100 µL of the primary stain to each well. Store the 24-well plate in a 4°C refrigerator overnight.
8. Wash another two times with PBS.
9. Add 100 µL of the secondary stain to each well. Incubate the 96-well plate on a rocker in an incubator at 37°C and 5% CO_2 for 20 minutes. After 20 minutes, aspirate the stain.
10. Wash another two times with PBS.
11. Image the cells using fluorescent (UV) microscopy.

[20] These solutions should not be prepared at the same time as the first three, but rather the day they are to be added (see the staining procedure). The optimal concentrations for each has yet to be determined.
[21] This step must be done in the sterile tissue culture hood to protect the integrity of the stain.

Cell Culture Media for OSU2 Cells

Ed. Alex Hissong 01/09/11

Jessica Winter 07/13/2007

Rev 4

Purpose

This protocol describes methods to make media for the culture of OSU2 cells.

Materials

DMEM/F-12 (Invitrogen),	89 mL
Pen/Strep[22] [Invitrogen/Gibco 15140-148],	1 mL
FBS (Fetal Bovine Serum) [Sigma F6178]	11 mL

100 mL sterile bottle

25 mL sterile pipette

70 % Alcohol

Safety Concerns and PPE:

Cell culture media must be synthesized in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device.

[22] There are two concentrations 5000 and 10000 units. Usually these are diluted to 100 U/100 mL media (1%). Higher concentrations (2%) may be used for the culture of primary cells.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When making media all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure

Media Preparation (100 ml)

1. Add 97 ml of DMEM/F-12 to a sterile 100 ml pre-sterilized container in the tissue culture hood.
2. Add 2 ml of FBS to container. Sera should be thawed in advance, but cannot last in the refrigerator for more than a few weeks. If necessary, sera can be thawed in the water bath on the day of use, but it is better to thaw the vial in the refrigerator a few days in advance of use. If opening a new vial, triturate (mix using the pipette) several times to ensure that proteins at the bottom of the vial are evenly distributed.
3. Add 1 ml of pen-strep (penicillin-streptomycin).
4. Media is now ready for use.

Cell Culture Media for Rat Astrocyte Cells

Joe Grodecki 5/25/2012

Rev 0

Purpose

This protocol describes methods to make media for the culture of Rat Astrocyte cells.

Materials

DMEM/High Glucose 1X (Invitrogen),	84 mL
Pen/Strep[23] [Invitrogen/Gibco 15140-148],	1 mL
FBS (Fetal Bovine Serum) [Sigma F6178]	15 mL
100 mL sterile bottle	
25 mL sterile pipette	
70 % Alcohol	

Safety Concerns and PPE:

Cell culture media must be synthesized in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.

[23] There are two concentrations 5000 and 10000 units. Usually these are diluted to 100 U/100 mL media (1%). Higher concentrations (2%) may be used for the culture of primary cells.

- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When making media all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure

Media Preparation (100 ml)

5. Add 84 ml of DMEM/ High Glucose 1X to a sterile 100 ml pre-sterilized container in the tissue culture hood.
6. Add 15 ml of FBS to container. Sera should be thawed in advance, but cannot last in the refrigerator for more than a few weeks. If necessary, sera can be thawed in the water bath on the day of use, but it is better to thaw the vial in the refrigerator a few days in advance of use. If opening a new vial, triturate (mix using the pipette) several times to ensure that proteins at the bottom of the vial are evenly distributed.
7. Add 1 ml of pen-strep (penicillin-streptomycin).
8. Media is now ready for use.

Passaging Cells

Jessica Winter

07/13/07

Rev 3

Purpose: Cells that divide will continue to fill up a culture dish or flask overwhelming the resources. It is important to split the cells periodically to reduce numbers in each flask, and allow better access to cell culture medium.

Materials:

Culture Dishes (usually 10 cm diameter Petri dishes or 75 cm^2 T-flasks for propagating a culture)

Phosphate buffered saline (PBS) without Calcium and Magnesium [Sigma P3813]

Trypsin-EDTA (for highly adherent cells only) [Sigma T4049]

Cell Culture Medium

15 ml sterile conical tube

Sterile Pipettes (5 and 10 ml)

Safety Concerns and PPE:

Cells must be passaged in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device. In addition, counting cells exposes the user to biohazards. Trypsin/EDTA (TRED) is a protease meaning it dissolves proteins. Operators should wear gloves to protect against this hazard.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When passaging all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure for Adherent Cells:

25. EtOH wash required pipettes, medium, TRED, conical, and PBS and place in hood.
26. In sterile culture hood, aspirate medium using sterile pasteur pipette.
27. Add 3 ml PBS,[24,25] swirl dish to coat, and aspirate with sterile pasteur pipette.
28. *If cells are highly adherent (e.g., fibroblast) only*, add 2 ml trypsin-EDTA (TRED)[26]. Observe in microscope for 2-3 minutes, until cells exhibit a rounded morphology and begin to detach from dish. If possible, aspirate TRED.[27]
29. Add 8 ml culture medium,[28] and starting at one corner of the dish, wash the surface of the dish from the middle toward the outside. Rotate the dish until all surfaces have been washed.
30. Aspirate medium and cells into a sterile 15 ml conical tube.
31. Centrifuge at 3000-4000 RPM for 3 minutes at 25 ºC.
32. Without disturbing cell pellet, carefully aspirate as much media as possible.
33. Resuspend cells in desired volume,[29] and pipette to new cell dishes.
34. Add medium to bring total volume to 10 ml.[30]

Procedure for Non-Adherent Cells:

1. In sterile culture hood, remove appropriate amount of cells[31] using sterile transfer pipette.
2. Add medium to bring to recommended total volume.[32]

[24] PBS without Ca and Mg is added to rinse away remaining Ca ions and debris. Ca is important in cell adhesion, and its removal will aide isolation of the cells.
[25] 3 ml for 10 cm dish. Otherwise use about 1/3 the volume used for feeding.
[26] Trypsin is a protease that digests cell connections to the dish. EDTA is a chelator that will bind remaining Ca ions, inhibiting cell attachment.
[27] It used to be standard to rap the sides of the dish to detach cells at this stage. This is now viewed negatively as it can cause significant cell aggregation.
[28] For 10 cm dish, else 80% of feeding volume.
[29] See recommendation of company from which cells were purchased. For example, ATCC recommends 1:4 passage for PC12 cells. I would suspend cells in 8 ml, and add 2 ml to each of 4 dishes.
[30] Or appropriate volume for size of dish used.
[31] See recommendation of company from which cells were purchased. For example, if ATCC recommends 1:4. Remove cells and medium and split between four flasks, equally.

[32] For example, for 75 cm^2 t-flask, ~30 ml. Check with supplier for recommended volume and feeding procedures.

Passaging Rat Astrocyte Cells

Jessica Winter

07/13/07

Rev 3

Joe Grodecki

Ed 5/25/12

Purpose: Cells that divide will continue to fill up a culture dish or flask overwhelming the resources. It is important to split the cells periodically to reduce numbers in each flask, and allow better access to cell culture medium.

Materials:

Culture Dishes (usually 10 cm diameter Petri dishes or 75 cm^2 T-flasks for propagating a culture)

Phosphate buffered saline (PBS) without Calcium and Magnesium [Sigma P3813]

StemPro Accutase [Life Technologies]

Cell Culture Medium

15 ml sterile conical tube

Sterile Pipettes (5 and 10 ml)

Safety Concerns and PPE:

Cells must be passaged in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device. In addition, counting cells exposes the

user to biohazards. StemPro Accutase (TRED) is a protease meaning it dissolves proteins. Operators should wear gloves to protect against this hazard.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.

PPE: When passaging all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure for Adherent Cells:

35. EtOH wash required pipettes, medium, TRED, conical, and PBS and place in hood.
36. In sterile culture hood, aspirate medium using sterile pasteur pipette.
37. Add 3 ml PBS,[33,34] swirl dish to coat, and aspirate with sterile pasteur pipette.
38. *If cells are highly adherent (e.g., fibroblast) only*, add 2 ml StemPro Accutase. Observe in microscope for 20-30 minutes, until cells exhibit a rounded morphology and begin to detach from dish. If possible, aspirate StemPro Accutase.[35]
39. Add 8 ml culture medium,[36] and starting at one corner of the dish, wash the surface of the dish from the middle toward the outside. Rotate the dish until all surfaces have been washed.
40. Aspirate medium and cells into a sterile 15 ml conical tube.
41. Centrifuge at 250 × g for 5 minutes at 25 ºC.
42. Without disturbing cell pellet, carefully aspirate as much media as possible.
43. Resuspend cells in desired volume,[37] and pipette to new cell dishes.
44. Add medium to bring total volume to 10 ml.[38]

[33] PBS without Ca and Mg is added to rinse away remaining Ca ions and debris. Ca is important in cell adhesion, and its removal will aide isolation of the cells.
[34] 3 ml for 10 cm dish. Otherwise use about 1/3 the volume used for feeding.
[35] It used to be standard to rap the sides of the dish to detach cells at this stage. This is now viewed negatively as it can cause significant cell aggregation.
[36] For 10 cm dish, else 80% of feeding volume.
[37] See recommendation of company from which cells were purchased. For example, ATCC recommends 1:4 passage for PC12 cells. I would suspend cells in 8 ml, and add 2 ml to each of 4 dishes.
[38] Or appropriate volume for size of dish used.

Thawing Cells

Jessica Winter

07/13/2007

Rev 2.

Purpose: This protocol describes the general procedure for thawing cells from frozen stocks. Dilution quantities given here are for a frozen stock of 1 x 10^6 cells/ml. ATCC stocks may recommend different dilution ratios. FOLLOW ATCC INSTRUCTIONS when they vary from those given here.

Materials:

Frozen cells

100 ml beaker of 37 ºC water

Sterile 1 ml plastic pipette

2- Sterile 10 ml plastic pipettes

Sterile 15 ml conical

Sterile Pasteur pipettes

Centrifuge

Prepared Cell culture dish

Cell Culture medium

Safety Concerns and PPE:

Cells must be thawed in a sterile environment. Note that a sterile environment DOES NOT provide protection against chemical hazards. If chemical hazards are present work should be performed with a respirator or similar protective device. Operators are also exposed to cryohazards when freezing cells. Please read the SOP on dealing with cryoagents before proceeding.

- Operators must perform indicated steps in the sterile, laminar flow tissue culture hood.
- Wipe all surfaces with 70% ethanol.
- Work as far back in the hood as possible.
- Avoid reaching over all sterile surfaces.
- When thawing, operators should avoid splashing of cryogenic fluids.

PPE: When thawing all operators should wear cyrogenic gloves, lab coat, and safety glasses. Latex or nitrile gloves should be worn when handling thawed samples.

Procedure:

1. Warm medium to 37 ºC for 10-15 minutes.
2. Prepare hood. Turn on blower and lights, wipe down with 70% EtOH. Open Pasteur pipette box.
3. Wipe down 1 ml pipette, 15 ml conical, and cell culture medium and place in hood.
4. Place prepared cell culture dish in hood.
5. Using 10 ml pipette, remove 10 ml of warm cell culture medium and place in 15 ml conical.
6. Obtain 37ºC water from water bath using 100 ml beaker.
7. Place vial to be thawed in 37 ºC water without submerging cap.[39,40]
8. Hold cells in water until thawed.

[39] Carry water to cell location to minimize exposure to other temperatures.
[40] Cells will either arrive on dry ice or will be stored in – 80 freezer.

9. When thaw is complete, wipe down vial and place in hood.
10. Crack the cap of the vial.
11. Using 1 ml pipette, transfer cells to 10 ml of warmed medium.
12. Allow to sit in warm medium for 2-3 minutes.[41]
13. Close down cap securely. Centrifuge cells for 3 minutes at 4000 at room temp. This will create a pellet of cells at the bottom of the centrifuge tube.
14. Return tube to hood, crack cap, and aspirate cell culture medium from 15 ml conical using a sterile Pasteur pipette. [42] Be careful not to aspirate or disturb cell pellet!
15. Using 10 ml pipette, add 10 ml warm cell culture medium to cell pellet.
16. Then using same pipette, slowly triturate the cell pellet to break it up and reduce clumps.
17. When cells have been sufficiently mixed, add all 10 ml to prepared cell culture dish.
18. Place in incubator at 37 ºC, or ATCC recommended temperature.

[41] This step allows cells to thaw before being exposed to the trauma of the centrifuge.
[42] This step removes any DMSO that may remain in solution.

Sterile Tissue Culture Hood Technique (Vertical Laminar Flow)

John Larison

Rev 1

07/24/2007

Purpose: The vertical laminar flow of the sterile tissue culture hood allows for working with hazardous organisms without being exposed to aerosols. The filter that removes these particulates from the air also makes the hood an appropriate environment for tissue culture by preventing contamination of tissue culture dishes and sterile solutions such as stock or medium.

Materials:

Sterile Tissue Culture Hood

Materials required for sterile procedure (Autoclaved as necessary)

70% Ethanol

Kimwipes

Safety Concerns and PPE:

When working with biohazardous materials, the operator risks contamination during procedural work.

- Operators should wear closed shoes, gloves, lab coat (back opening if possible), and safety glasses.
- Wearing a face shield, surgical mask, or respirator will further prevent airborne contamination.
- Never wear jewelry (such as watches, bracelets, and rings) which can act as a vector.

The sterile tissue culture hood sterilizes by UV light, posing radiation hazards.

- The hood shield is UV protected. Only turn on the UV light when the hood shield is completely lowered.

70% Ethanol is a flammable solvent, and when combined with heat sources (i.e., alcohol lamp for polishing pipet tips), presents a combustion hazard.

PPE: When using the sterile tissue culture hood all operators should wear latex or nitrile gloves, lab coat, and safety glasses.

Procedure (Standard Items):

1. Wash hands and arms before putting on lab coat and gloves.
2. After putting on personal protective equipment, wipe hands with 70% ethanol[43] and spray sleeves of lab coat as well.
3. Open hood shield up to the arrow.
4. Turn on the visible light[44], outlets, and blower[45] by flipping the switches on the right side of the hood.
5. Wipe the area inside the hood with 70% ethanol, working from back to front.
6. Turn on vacuum pump if necessary[46].
7. Wipe all materials with 70% ethanol before placing in the hood[47].

[43] This should be done every time before entering the hood.

[44] The UV light may be turned on 5 minutes before use (and before the hood shield has been raised) for extra prevention of contamination

[45] The blower should never be turned on before the hood shield has been raised. This can damage the motor.

[46] The vacuum can be accessed in the hood by turning the valve on the right wall.

[47] Larger items should be placed as far back as possible to avoid disrupting the airflow. No items should ever be placed on the metal grill at the front of the hood. Operators may choose to wait two minutes after placing necessary items in the hood and before beginning work to allow the blower to remove any airborne contaminants from the utensils

8. Unscrew all bottle caps but leave caps on. Release caps on tubes[48].
9. Work in the center of the hood, near the back, avoiding reaching over any sterile surfaces.
10. When finished, place all unwanted biological material in the biowaste bag[49].
11. Wipe down all surfaces with 70% ethanol.
12. If vacuum was used for aspiration, tube must be sterilized as well using 70% ethanol.
13. Label (with identification, name, and date) and properly store all wanted biological material.
14. Wipe gloves with 70% ethanol, then dispose of gloves in the biowaste bin.
15. Turn off the outlets and blower[50].
16. Lower the hood shield.
17. Turn the light switch to UV, and allow it to sterilize the hood for at least 20 minutes after use.
18. Glassware should be cleaned by rinsing once with sodium hypochlorite solution (bleach 1:10 solution with water), once with soapy water, then three rinses with distilled, deionized water.

Procedure (Sterile Pipet Technique):

1. Sterile pipets (wrappers) should be sprayed with 70% ethanol before being placed in the sterile tissue culture hood[51].
2. The wrapper should not be removed until the pipet is in the hood and is ready to be used.
3. Grip the pipet only near the top when necessary, and never allow the bottom tip of the pipet to ever touch any surface[52].
4. Use one pipet per solution.
5. When pipet is not in use, keep bottom tip at higher elevation than the top of the pipet without holding it over another sterile surface.
6. When finished, pipets may be discarded in the biowaste bag.

Procedure (Bottles):

[48] If lids have to be removed completely, tilt on another dish to minimize contact with the hood surface. Always place caps facing down.

[49] When the bag becomes full, it must be autoclaved, then disposed of in the biowaste bin. See biological waste disposal SOP for more details.

[50] To ensure decontamination, the operator may choose to leave the blower running for five minutes after finishing.

[51] Pipets without graduated markings (Pasteur pipettes) have already been autoclaved and may be found in the metal canister in the hood. Extra canisters are stored in the wooden cabinet.

[52] This includes the sides and necks of any bottles.

1. Make sure solution is thoroughly mixed[53].
2. Twist cap off carefully with thumb and index finger[54].
3. Hold cap with fingers while pipetting if possible. If cap must be set down, place it face (open side) down.
4. When finished pipetting, put the cap back on the bottle with thumb and index finger only.

Procedure (Tissue Culture Dishes):

1. While working, hold lid between thumb and index finger and tilt the dish toward you to keep your other hand free to pipet and to help keep your hands from being directly over open dishes. (Clam shell technique)
2. Label the dishes with identification, passage number, name, and date.

[53] Never invert the bottle to mix a solution. Always swirl.
[54] Never reach over an open bottle.

Operation of Vacuum Pumps

Jessica Winter

Rev. 1

07/18/2007

Purpose: Vacuum pumps are used to supply vacuum to vacuum systems.

Materials:

Vacuum pump

Hose/attachments

Vacuum Oil (for pump maintenance)

Dry ice/Acetone or Liquid Nitrogen (cryoagent)

Safety Concerns and PPE:

Vacuum pumps present pressure hazards to the operator. Vacuum pumps must be used with a cold trap to condense and contain any released vapors, which can be extremely damaging to the pump. Cold traps require the use of cryoagents (Dry ice/acetone slurry or liquid nitrogen) which present a burn hazard to the user. To safely address these hazards:

- Always operate the vacuum oven with a cold trap to condense and retain vapors. Either a dry ice/acetone slurry (-78 C) or liquid nitrogen (-196 C) may be used. See specific information on cold traps below for more information.
- Always use cryoprotective gloves when handling cryoagents.
- Wear lab coat and safety glasses to prevent against splash/spill hazards.

If liquid nitrogen is the coolant, liquid air can condense in the trap, inviting explosion. Liquid air, comprising a combination primarily of oxygen and nitrogen, is warmer than liquid nitrogen. Depending on the nitrogen content, air liquefies anywhere from -190°C (-310°F) (5°C warmer than liquid nitrogen) to -183°C (-297.4°F) (liquid oxygen). If liquid nitrogen is used, the trap should be charged only after the system is pumped down lest a considerable amount of liquid oxygen condenses, creating a major hazard.

Handle any liquid gas carefully; at its extremely low temperature, it can produce an effect on the skin similar to a burn. Moreover, liquefied gases spilled on a surface tend to cover it completely and intimately, and therefore cool a large area.

The evaporation products of these liquids are also extremely cold and can produce burns. Delicate tissues, such as those of the eyes, can be damaged by an exposure to these cold gases which is too brief to affect the skin of the hands or face.

Eyes should be protected with a face shield or safety goggles (safety spectacles without side shields do not give adequate protection). Gloves should be worn when handling anything that is or may have been in contact with the liquid; insulated gloves are recommended, but leather gloves may be used. The gloves must fit loosely so that they can be thrown off quickly if liquid should spill or splash into them. When handling liquids in open containers, high-top shoes should be worn with trousers (cuffless if possible) worn outside them.

Stand clear of boiling and splashing liquid and its issuing gas. Boiling and splashing always occur when charging a warm container or when inserting objects into the liquid. Always perform these operations SLOWLY to minimize boiling and splashing.

Should any liquefied gas used in a cold trap contact the skin or eyes, immediately flood that area of the body with large quantities of unheated water and then apply cold compresses. Whenever handling liquefied gases, be sure there is a hose or a large open container of water nearby, reserved for this purpose. If the skin is blistered, or if there is any chance that the eyes have been affected, take the patient immediately to a physician for treatment.

Oxygen is removed from the air by liquid nitrogen exposed to the atmosphere in an open Dewar. Store and use liquid nitrogen only in a well ventilated place; owing to evaporation of nitrogen gas and condensation of oxygen gas, the percentage of oxygen in a confined space can become dangerously low. When the oxygen concentration in the air becomes sufficiently low, a person loses consciousness without warning symptoms and will die if not rescued. The oxygen content of the air must never be allowed to fall below 19%.

The appearance of a blue tint in liquid nitrogen is a direct indication of its contamination by oxygen, and it should be disposed of, using all the precautions generally used with

liquid oxygen. Liquid nitrogen heavily contaminated with oxygen has severe explosive capabilities. In addition, an uninsulated line used to charge Dewars will condense liquid air; liquid air dripping off the line and revaporizing causes an explosive hazard during the charging operation.

If the cold trap mixture is allowed to freeze, and the cold trap becomes rigid, slight movement in other parts of the apparatus could result in breakage of the trap or other glassware.

If a gas trap has to be lifted out of the Dewar cold bath for inspection, it will be difficult to reinsert into the slush. Therefore, it is preferable to use a liquid that will not freeze at -78.5 °C.

PPE: The operator should wear safety gloves, lab coat, and safety glasses. When filling the cold trap use cryoresistent gloves. In addition, the vacuum oven should be operated in the chemical fume hood to prevent accidental exposure to chemical vapors.

Procedure:

1.) Check Oil level.
    a. Maintain oil level between 1/3 and 2/3 in the sight glass.
    b. Monitor oil level immediately after starting as it will fluctuate slightly, and will change as a function of oil temperature.
2.) Ensure the suction of the pump is connected to a cold trap prior to connection to operation. All vacuum pumps should be used with a cold trap except for filtration pumps and oil-less pumps.

3.) Ensure the outlet of the cold trap is connected to the apparatus that will be evacuated.
4.) Immerse cold trap to a point that is below the o-ring joint (approximately ¾ of the lower section of the vacuum trap) in a dewar containing a cryoagent.
5.) Ensure the vacuum pump outlet is unobstructed, and directed to a fume hood.
6.) Start the vacuum pump.
    a. Monitor oil level until the level stabilizes.
        i. If the level drops out of the sight glass at anytime immediately stop the vacuum pump.
        ii. Determine the cause of the low oil level.
        iii. Add oil to a level which half fills the oil sight glass.
    b. Observe the pressure lowering indicating the proper pump operation.
7.) Monitor Oil level during operation and liquid nitrogen / dry ice level in the dewar.

Specific Information on Cold Traps
(http://chemistry.osu.edu/ehs/handbook/gases/coldtrap.htm)

Cold traps are used in instrumentation and elsewhere to prevent the introduction of vapors or liquids into a measuring instrument from a system. A cold trap provides a very low temperature surface on which such molecules can condense, and improves pump-down by one or two magnitudes.

However, cold traps improperly employed can impair accuracy, destroy instruments or systems, and be a physical hazard. For example, many of the slush mixtures used in cold traps are toxic or explosive hazards, and this is not indicated in the literature.

The equilibrium point of a dry-ice-acetone slush is -78°C (-108.4°F), which, does not remove water vapor; a temperature of at least -100°C (-148°F) is required to eliminate water vapor, or, alternatively, exposure to anhydrous phosphorus pentoxide (P_20_5). This material is usually rejected for field use because of possible biological, fire and explosive hazards: in absorbing water it produces heat, and reacts vigorously with reducing materials.

Vapor pressure of most standard roughing pump oils is 10^{-3} to 10^{-4} torr at 25°C (77°F), 1/5 of this value at 0°C, and negligibly small below the temperature of dry ice. Fractionating oils currently used in vacuum pumps have very low vapor pressures, ranging from 20 x 10^{-6} to 10^{-7} torr, and pose no problem for most work. Nevertheless, gases produced by thermal decomposition of the oil may contaminate the vacuum unless trapped.

VIRTUAL LEAKS

If the cold trap is chilled too soon after the evacuation of the system begins, gases trapped will later re-evaporate, when the pressure reaches a sufficiently low value. The evaporation of the refrigerated and trapped gases is not rapid enough to be evacuated by the system, but is enough to degrade the vacuum, producing symptoms very similar to those of a leak.

To avoid these virtual leaks, keep the traps warm until a vacuum of about 10^{-2} torr is obtained. The tip of the trap is then cooled until ultimate vacuum is reached, at which time the trap may he immersed in the coolant to full depth.

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