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DETERMINING IF MICROCHIMERIC FETAL CELLS CARRY THE PATERNALLY-INHERITED GENOME IN THE MATERNAL BRAIN

By

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A capstone project submitted for Graduation with University Honors

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APPROVED

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ABSTRACT

Microchimerism occurs when cells of one organism are acquired by another organism, and this primarily occurs during pregnancy due to the intimate exchange of molecules between mother and fetus via the placenta. There is evidence that fetal microchimeric cells translocate to the maternal brain during gestation, with the potential for long-term persistence. However, the effects of these cells on mothers remain unknown. Prior work in my PI's lab found correlative evidence for an effect of embryos' paternally-inherited genome on gene expression in the maternal medial preoptic area (MPoA), a brain region critical to maternal behavior and physiology. My research investigates whether fetal microchimeric cells in the maternal brain could provide the causal mechanism for this currently unexplained effect of fathers on mothers. My first step was crossing wild-type female mice to males that carry a GFP reporter gene and using a cryostat to slice the brains and immunohistochemistry to determine whether GFP-labeled fetal cells are detectable in postpartum female brains under a UV microscope. Then, I conducted additional crosses within and between genetically distinct strains. Although the antibody staining required additional optimizations, I was able to conclude, based on native GFP fluorescence, that we did not detect any GFP+ microchimeric fetal cells in the maternal brain.

ACKNOWLEDGMENTS

I would like to extend my deepest gratitude to Dr. Campbell and the Campbell Lab Crew for your unwavering patience and compassion. Thank you for harboring a welcoming and humble atmosphere to discuss science.

It is with the utmost gratitude that I express my thanks to my parents for their constant love and support. Thank you for teaching me your work ethic and for supporting me through every academic and personal endeavor. I dedicate all of my accomplishments to you, and this is part of your legacy.

Thank you to my brothers who always keep me humble and surround me with virtues of laughter and sibling love. Malek taught me how to lead with kindness and determination, and Sami made me want to become the best big sister and role model. I hope I make you proud.

And to the people in my life who are great friends and may as well be family, Alexander, Asha, Ivy, Mowkthika, Ruth, Samiksha, and Sofia, thank you for celebrating my triumphs and nurturing me through my frustrations. I appreciate you all endlessly.

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INTRODUCTION

Pregnancy is not confined to the uterine walls, as it affects the mother's entire body. The placenta, a vital organ during pregnancy, serves as the interface between the mother and the developing fetus. It facilitates the exchange of molecules and cells, allowing for the passage of waste products from the fetus to the mother, while essential nutrients and oxygen are directed from the mother to the developing fetus. This intimate interaction results in a phenomenon known as microchimerism, wherein cells from one organism translocate and establish themselves within another organism, despite their genetic differences.

Fetal Acquisition of Maternal Microchimeric Cells

The acquisition of maternal microchimeric cells by the developing fetus introduces a unique immunological dynamic. Maternal cells carrying specific antigens can interact with the fetal immune system, potentially influencing immune tolerance and response. These interactions are thought to contribute to fetal immunity, helping to shape the developing immune system and establish tolerance to maternal antigens (Naik et al., 2019). Studies have shown that maternal microchimeric cells can be found in various fetal tissues, including the liver, spleen, thymus, and bone marrow, suggesting their potential role in immune cell development and regulation (Naik et al., 2019).

It has been proposed that maternal microchimeric cells may contribute to the establishment of immune tolerance and protection against certain immune-mediated disorders in the fetus (Kinder et al., 2017). The presence of maternal T cells, for example, has been associated with a reduced risk of conditions such as allergic diseases and autoimmune disorders in childhood (Kinder et al., 2017). Furthermore, studies have indicated that maternal microchimeric cells can participate in immune responses and play a role in fetal defense against

infections in their environment (Kinder et al., 2017).

The presence of maternal microchimeric cells in the developing fetus raises intriguing questions about their potential impact on fetal development. While the exact mechanisms remain largely unknown, studies have suggested that maternal microchimeric cells may have the capacity to differentiate into various cell types and integrate into fetal tissues (Naik et al., 2019). This phenomenon, known as cell fusion or transdifferentiation, could potentially contribute to tissue repair and regeneration during fetal development (Naik et al., 2019).

Additionally, maternal microchimeric cells have been found to secrete factors such as growth factors and cytokines, which could modulate the local microenvironment and influence cell proliferation, differentiation, and organ creation (Jeanty et al., 2014). It is plausible that maternal microchimeric cells may play a role in the regulation of fetal growth, tissue maturation, and organ development, although further research is needed to identify the precise mechanisms and extent of their involvement (Jeanty et al., 2014).

The long-term consequences of maternal microchimeric cell acquisition in fetuses remain an active area of research and debate. Some research has suggested potential associations between maternal microchimeric cells and certain health outcomes later in life; for instance, maternal cells have been detected in the brains of individuals with neurodevelopmental disorders, such as autism spectrum disorders, raising the possibility of a link between maternal microchimeric cells and neurodevelopmental abnormalities (Bülent & Osman, 2019). However, the causative relationship and underlying mechanisms have yet to be fully established.

Moreover, the persistence of maternal microchimeric cells in the fetal body beyond pregnancy raises questions about their potential influence on the individual's health throughout their lifespan. Maternal microchimeric cells have been detected in various tissues and organs,

including the bone marrow, liver, and epithelial tissues (Naik et al., 2019). The interaction between maternal microchimeric cells and the fetus's immune system, as well as their interactions with the fetal cells, may have implications for autoimmune diseases, cancer development, and tissue regeneration (Boddy et al., 2015).

The acquisition of maternal microchimeric cells in fetuses represents an intriguing aspect of fetal development with potential implications for immune modulation, fetal development, and long-term health outcomes.

Maternal Acquisition of Fetal Microchimeric Cells

Fetal microchimerism gained significant scientific attention in the twentieth century when researchers developed techniques to detect and analyze fetal DNA present in the maternal bloodstream (Cómitre-Mariano et al., 2022). This breakthrough provided a foundation for exploring the mechanisms underlying the translocation and detection of fetal microchimeric cells.

Detecting fetal microchimeric cells in the maternal body presents unique challenges due to their low frequency and the need to differentiate them from host cells. Various techniques have been employed to identify and characterize fetal microchimeric cells, each with its own advantages and limitations. A frequently employed method, known as immunohistochemistry, utilizes antibodies that target specific antigens, like the green fluorescent protein found only in GFP+ cells, in order to visualize and isolate any transgenic fetal microchimeric cells that encode for GFP. However, optimizing antibody staining protocols and exploring alternative antibodies are necessary to improve detection sensitivity. Another method involves the amplification of genetic markers associated with fetal cells through polymerase chain reaction (PCR) analysis. This approach allows for the identification of paternally-inherited genes or the presence of

marker genes unique to fetal cells. The widely used SRY gene, found exclusively on the Y chromosome in males, serves as a valuable marker for detecting male-derived fetal microchimeric cells. Additionally, by employing fluorescence-activated cell sorting (FACS), researchers can effectively identify and isolate fetal microchimeric cells that have paternally-inherited GFP expression based on their distinct fluorescent properties, providing valuable insights into their presence and behavior within the maternal body.

Microchimerism is a prevalent occurrence in human pregnancy, and its impact on maternal health has been studied extensively. Research has revealed both positive and negative health effects associated with the acquisition of microchimeric cells by the mother. Evidence suggests that fetal cells can translocate to various maternal tissues, including the brain, thyroid, and breasts (Boddy et al., 2015). The exact function of these cells and the mechanisms by which they translocate remain largely unknown. However, studies have linked the presence of fetal microchimeric cells in autoimmune disease sites, such as the thyroid gland and joints, to the triggering of autoimmune diseases and even cancer (Boddy et al., 2015). The acquisition of fetal cells by the mother occurs early in pregnancy, meaning that even in cases of pregnancy termination or loss, the mother likely retains a population of microchimeric cells (Sunami et al., 2010). These cells can persist in a woman for decades, potentially throughout her lifetime (Fujimoto et al., 2021). Furthermore, it has been observed that younger siblings can acquire cells from older siblings via the mother, perpetuating the presence of microchimeric cells across multiple pregnancies (Nørgaard-Pedersen et al., 2021).

The presence of fetal microchimeric cells can provoke an autoimmune response, leading to the development of autoimmunity against the mother's own cells. Additionally, these cells have been associated with the onset or exacerbation of autoimmune diseases such as rheumatoid

arthritis, cancer, and lupus (Naik et al., 2019). However, microchimeric cells have also demonstrated the potential for tissue regeneration and repair.

Fetal microchimeric cells possess the ability to cross the blood-brain barrier, and their presence within the maternal brain has been associated with both positive and negative effects on maternal cognitive function. Due to their differentiating capabilities, fetal microchimeric cells have the potential to enhance brain function and aid in the repair of damaged brain cells and tissues. Some studies have even shown that the presence of these cells correlates with a reduced risk of developing Alzheimer's Disease, possibly attributed to their capacity to differentiate into various cell types, including neurons, which can aid in the repair of damaged brain cells (Chan & Nelson, 2013). Conversely, the persistence of these cells has been suggested to increase the risk of certain neurological disorders in mothers, including schizophrenia, bipolar disorder, and depression (Bülent & Osman, 2019). This may be due to an inflammatory autoimmune response within the maternal brain triggered by the presence of these foreign cells.

The impact of fetal microchimeric cells on maternal longevity is an area of ongoing scientific investigation. While there is no definitive evidence to suggest that fetal microchimeric cells directly contribute to women living longer than men, some studies have explored potential associations between microchimerism and health outcomes (O'Donoghue, 2008). It is hypothesized that fetal cells may exert immunomodulatory effects on the mother, potentially influencing her health and longevity. Some studies have suggested that microchimeric cells could have both beneficial and detrimental effects on maternal health, depending on various factors such as the specific cell types involved, the maternal immune response, and the context of disease or health conditions (O'Donoghue, 2008). For example, it has been proposed that fetal microchimeric cells might contribute to tissue repair and regeneration in the maternal body

(O'Donoghue, 2008). These cells have been observed to differentiate into various cell types, including those involved in tissue repair, in animal studies (O'Donoghue, 2008). This regenerative potential could potentially have positive effects on maternal health and longevity. However, more research is needed to establish the extent and significance of these effects. The impact of microchimerism on the development and progression of such conditions is still not fully understood and requires further investigation (O'Donoghue, 2008).

The consequences of acquiring microchimeric cells from multiple individuals and their implications for maternal health are not yet fully understood. However, studies have indicated that the acquisition of male cells by females can have negative effects on reproduction. A recent study revealed that approximately 79.1% of women who experience recurrent pregnancy loss have either an older brother or first-born son or both (Nørgaard-Pedersen et al., 2021). This may occur because men carry HY-specific T-cells that can negatively impact women's reproductive health (Nørgaard-Pedersen et al., 2021). The HY-specific T-cells are male-derived because they recognize and target antigens encoded by the Y chromosome only found in males, and they play an important role in the male immune response against certain cancers and infections (Teuscher et al., 2006). However, these cells can recognize and attack fetal cells that express paternally-inherited HY antigens, leading to pregnancy complications such as pregnancy loss and other reproductive health concerns (Nørgaard-Pedersen et al., 2021).

The study of microchimeric cells in women is an expanding and active field of research. Whether these male-derived cells affect maternal gene expression is largely unknown. Since fetuses inherit genes from both the mother and father, it is plausible that paternally inherited genes are expressed in the maternal brain as a result of fetal cell translocation to the brain.

With this research, I hope to determine whether fetal cells can carry paternally-inherited

genes to the maternal brain. If so, there will be new terrain to explore regarding how paternal genes will affect maternal brain function and behavior. It is possible that paternal gene expression in the maternal body that is in direct opposition to maternal gene expression is responsible for autoimmune diseases, such as rheumatoid arthritis, lupus, and cancers that were aforementioned in multiple studies. On the other hand, paternally-inherited genes not at odds with maternal gene expression may allow mothers to reap more regenerative benefits from fetal microchimeric cells. In either case, further research based on the results of this paper can produce remarkable gains in understanding the fetal-maternal microchimerism phenomenon.

METHODS

Cross-breeding and Brain Dissection

We crossed mice in order to test for evidence of fetal cell transfer to the brains of pregnant female mice. Female mice were weighed after mating to deduce whether the females were pregnant. Pregnant females were sacrificed by cervical dislocation in late gestation and brains were dissected into 4% paraformaldehyde in phosphate buffered saline (PBS).

For negative controls, C57BL/6J (hereafter, GFP-) male mice were crossed with GFP- females. Therefore, neither parent nor offspring should express the GFP protein. Positive controls consisted of C57BL/6-Tg(UBC-GFP)30Scha/J (hereafter, GFP+) male mice crossed with GFP+ females. In this case, parents and offspring should express GFP protein. Experimental crosses consisted of GFP+ males and GFP- females. Therefore, the offspring acquired the GFP allele from their fathers.

Fixing

Following at least 24 hours in paraformaldehyde at 4℃, brains were cryoprotected for 24-48 hours at 4℃ in a 30% sucrose solution made in 4% paraformaldehyde. The flash-freezing process began by removing the brains from the sucrose solution and cutting off the cerebellum. Afterward, the cerebrum was coated in optimal cutting temperature embedding medium (O.C.T.), to acclimate to the new medium. As the brain was acclimating in the O.C.T. for a few minutes, we filled around 75% of the cryomold with O.C.T. We removed the bubbles that were suspended in the medium before transferring the brain to the cryomold and submerging and embedding it. We marked the anterior side of the brain with a marker on its respective side on the mold to make brain mounting on the cryostat easier.

To begin cryofreezing, we filled a styrofoam container with dry ice and placed a beaker in the container. We added approximately 15mL of isopentane to the beaker and used forceps to lower the cryomold into the beaker, ensuring that the isopentane did not enter the cryomold. The O.C.T. in the cryomold immediately turned white as the flash-freezing began. More dry ice could be added along the perimeter of the beaker to speed up freezing time, as long as the dry ice did not touch the contents of the cryomold. The O.C.T. became fully opaque after 4-6 minutes, signaling the completion of freezing the embedded brain. Brains were stored at -80˚C until slicing.

Brain Slicing & Mounting

Once a brain was ready for slicing, we allowed it to acclimate to -20˚C for one hour to match the temperature of the Leica CM1860 cryostat. After one hour, we placed an even layer of O.C.T. onto the mount and let it acclimate to -20˚C so the O.C.T. froze. At this point, we removed the plastic cryomold from its frozen contents, and we placed a penny-sized amount of O.C.T on the frozen mount to act as an adhesive before immediately sticking the posterior side of the brain to the mount. We also sealed the perimeter of the frozen brain to the mount with O.C.T., and we placed the mounted brain back into the cryostat to allow the O.C.T. to freeze before locking it in place. After the brain was mounted into the machine, we locked the blade into place and begin slicing 40 μm coronal slices. We began collecting slices and continued collecting about 60 slices starting posterior to the nucleus accumbens (Figure 1a). The slices were placed into well plates that contained 1X PBS solution.

After the slices were acquired and acclimated to the PBS solution, we filled a petri dish with 1X PBS solution to begin the mounting process. We angled Fisherbrand Superfrost Plus microscope slides into the petri dish and used a paintbrush to gently remove the brain slices from

the well plates and maneuver them onto the slide (Figure 1b). About 15 slices were able to fit on each slide.

Slide Staining

Blocking could be done as soon as the brain slices had adhered to the slides. In order to begin this process, we dropped 5-6 drops of Normal Goat Serum (NGS) 10% in PBS on each slide. Then, we covered each slide with coverslips and incubated them in a humidified slide chamber for 1 hour. It is important to prevent the slides from drying from this point on and being exposed to light.

After blocking the slides for one hour, we needed to begin primary antibody staining. First, we slid off and discarded the coverslips on each slide and tapped off any excess blocking solution. Then we covered each slide with primary antibody solution, rabbit Polyclonal GFP antibody diluted 1:1000 in 1% bovine serum albumin (BSA) in PBS. We covered the slides with coverslips once more and incubated them in a humidified slide chamber at 4˚C overnight.

The last step of the staining procedure is coating the slides with a secondary antibody. We slid the coverslips off each slide and washed the slides in 1X PBS solution for ten minutes before covering each slide with a secondary antibody, Alexa Fluor® Goat Anti-Rabbit antibody diluted 1:1000 in 1% BSA in PBS. The slides were incubated for 30 minutes and washed in 1X PBS solution for another ten minutes. We tapped off excess liquid and added 2-3 drops of mounting medium, ProLong™ Diamond Antifade Mountant with DAPI, to each slide. Lastly, we placed a coverslip on each slide and used slight pressure to eliminate any bubbles in the slides before letting the slides cure in the dark overnight.

Under optimized conditions, a primary antibody would recognize the protein of interest, in our experiment this is GFP, and the secondary antibody would bind selectively to the primary

antibody. The secondary antibody would allow us to visualize the presence of the GFP proteins because the binding of the secondary antibodies to the primary antibodies would cause the secondary antibodies to activate and fluoresce if the primary antibodies bind to GFP proteins. The secondary antibody we used would fluoresce at a wavelength of 580 μm. Meanwhile, the mounting medium with DAPI would stain cell nuclei, which would discriminate between fluorescent background and whole cells. If native GFP, DAPI, and GFP antibodies fluoresced in the same region, then we could confirm that we found cells that are GFP+ and are present in the maternal brain.

Microscope

We used the Zeiss880 Inverted UV Spectral Airyscan microscope to detect any fluorescence. We used three lasers: one emitted 480 μm wavelengths (to detect GFP), another emitted 355 μm wavelengths (to detect DAPI), and the third emitted 580 μm wavelengths (to detect secondary antibodies). Through the eyepiece of the microscope and using 10X, 20X, and 40X with oil immersion lenses, we manually scanned the slides searching for any fluorescence, and we captured images with the aforementioned magnifications and using the Zeiss 2.3 software.

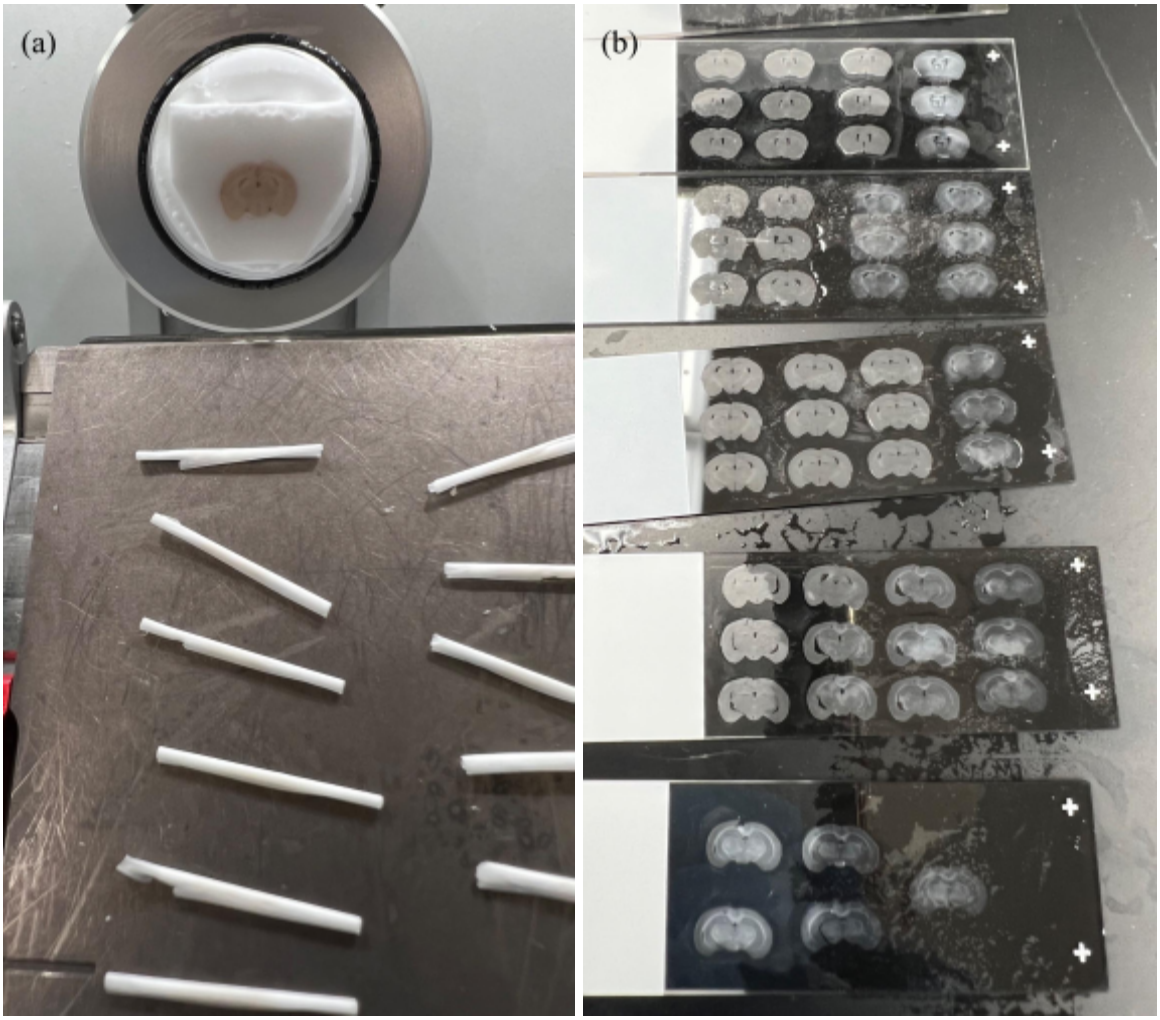

Figure 1. (a) The embedded and cryofrozen brain was mounted into the cryostat, and the slices remain frozen and rolled until they are dropped into the wellplates containing 1X PBS. (b) The slices were removed from the wellplates with a paintbrush before being mounted onto slides.

RESULTS AND DISCUSSION

Comprehending the Data

We used immunohistochemistry to test for evidence of fetal microchimeric cells in the brains of pregnant female GFP- mice crossed to GFP+ males. Using this approach, we were unable to detect any fluorescence that would indicate the translocation of GFP+ microchimeric cells to the maternal brain.

The DAPI mounting medium that was used to stain cell nuclei was successful, but the cell nuclei that fluoresced under DAPI fluorescent conditions did not colocalize with any native GFP fluorescence. Therefore, we can attribute any native GFP fluorescence in the negative controls and both trials to background fluorescence, rather than any GFP+ cells (Figure 2b-d).

Based on the positive control, the antibody staining required additional optimization because there is no visible signal for the GFP antibody in the GFP+ animals (Figure 2a). We would expect the GFP antibody to fluoresce because the GFP antibodies would be able to readily bind to GFP+ cells in the maternal brain. With this in mind, the most probable reason why the GFP antibody in Figure 2a did not fluoresce is that there was an issue with either the primary antibody binding to GFP and/or the secondary antibody binding to the primary antibody.

Unfortunately, we did not have time to accomplish the additional troubleshooting for optimizing the GFP antibody signal. However, although the antibody staining was uninformative, we are still able to make inferences based on the native GFP fluorescence. By comparing native fluorescence in both controls (Figure 2a-b) and subsequently comparing the native fluorescence in both experimental trials (Figure 2c-d) to the negative control, we see that the same level of background fluorescence is present in the negative controls and the experimental trials. In contrast, the positive controls have substantially more fluorescence, as we

would expect. Therefore, the similarities between the negative control and experimental animals indicate that, to the extent that it is possible to detect native GFP fluorescence without antibody staining, we did not find any evidence of fetal microchimerism in the forebrains of pregnant females.

Future Optimizations

Following our application of GFP antibodies, it appears that the antibody staining did not work, but we are still able to compare native GFP expression with our controls.
Using different antibodies or varying concentrations may allow for the detection of fetal microchimeric cells, indicating that the current approach does not definitively exclude their presence.

In order to optimize our GFP detection using primary and secondary GFP antibodies, we can attempt a series of dilutions of the primary and/or secondary antibodies with positive controls to identify the most ideal concentration of each type of antibody. By doing this, we would be able to troubleshoot the antibody staining in Figure 2a. Furthermore, we can try a different secondary antibody.

Besides optimizing the antibody staining, an entirely different approach can be used to detect GFP+ microchimeric fetal cells in the maternal brain. Rather than cryofreezing, slicing, and looking at the brains under a UV microscope to detect fluorescence, DNA from a maternal brain can be extracted and a marker gene can be amplified using PCR and gel electrophoresis. This method also allows us to identify a marker gene, only in a different manner. By comparing the banding patterns observed in experimental trials with those of both positive and negative controls, we can effectively determine the presence or absence of microchimeric fetal cells in maternal brains from hybrid crosses. This visual confirmation allows us to affirm or negate their

existence within the experimental samples. In addition, there are many marker genes we can amplify. We could amplify the GFP gene itself, or we could amplify genes that are male-derived, genes that are on the Y chromosome that would not otherwise be in these females. Such a gene that is commonly used as a marker for these purposes is the male-derived SRY gene. It is important to consider that if we do amplify male-derived genes, we would be doing so with the assumption that some offspring in the litter are male, which is a safe assumption to make because there are about 6-8 pups per litter. Therefore, it is a reasonable expectation that each litter should have at least one male. Amplification of the SRY gene for PCR and detection of fetal microchimeric cells in the maternal body has been accomplished by Gadi et. al (2008). In their research, they were able to use this technique to identify fetal microchimeric cells in breast tissue and peripheral blood of human female subjects. As a result of the increased number of such fetal microchimeric cells in the breast tissue of healthy subjects compared to those with breast cancer, they concluded that fetal microchimeric cells may have protective properties against breast cancer. The replication of this method on mice can involve two approaches. Firstly, it can be achieved by amplifying the SRY gene or other male-derived genes. Alternatively, hybrid crosses can be conducted between wildtype GFP- females and transgenic GFP+ males to enhance GFP amplification for PCR analysis.

Another approach that can be used is utilizing fluorescence-activated cell sorting (FACS). This technique sorts cells based on their characteristics, so we would be able to sort GFP+ microchimeric cells from GFP- maternal brain cells. FACS has been used in order to sort GFP+ fetal microchimeric cells from GFP- maternal cells located in wildtype GFP- female mice that were crossed with transgenic GFP+ male mice. The cells were acquired from maternal cardiac and skeletal muscle, and GFP+ fetal cells were detected in the damaged cardiac tissue. The

researchers were able to conclude that fetal cells can translocate to damaged maternal cardiac tissue and they can differentiate into “diverse lineages” (Kara et al., 2012).

Although we did not detect GFP+ presence from fetal microchimeric cells using fluorescent primary and secondary GFP antibodies, optimizing the concentrations and types of antibodies used or investigating other methodologies, such as PCR amplification of genetic markers or FACS, may produce varying results. Moreover, based on the native GFP expression in the positive control relative to the negative control and the lack of difference between the experimental and negative control, we did not detect native GFP expression in the experimental animals. We interpret any fluorescence present in both the negative control and experimental animals as background fluorescence. Our results are inconclusive until future optimizations of this method are conducted or alternative methods to detect paternally derived microchimeric cells have been attempted.

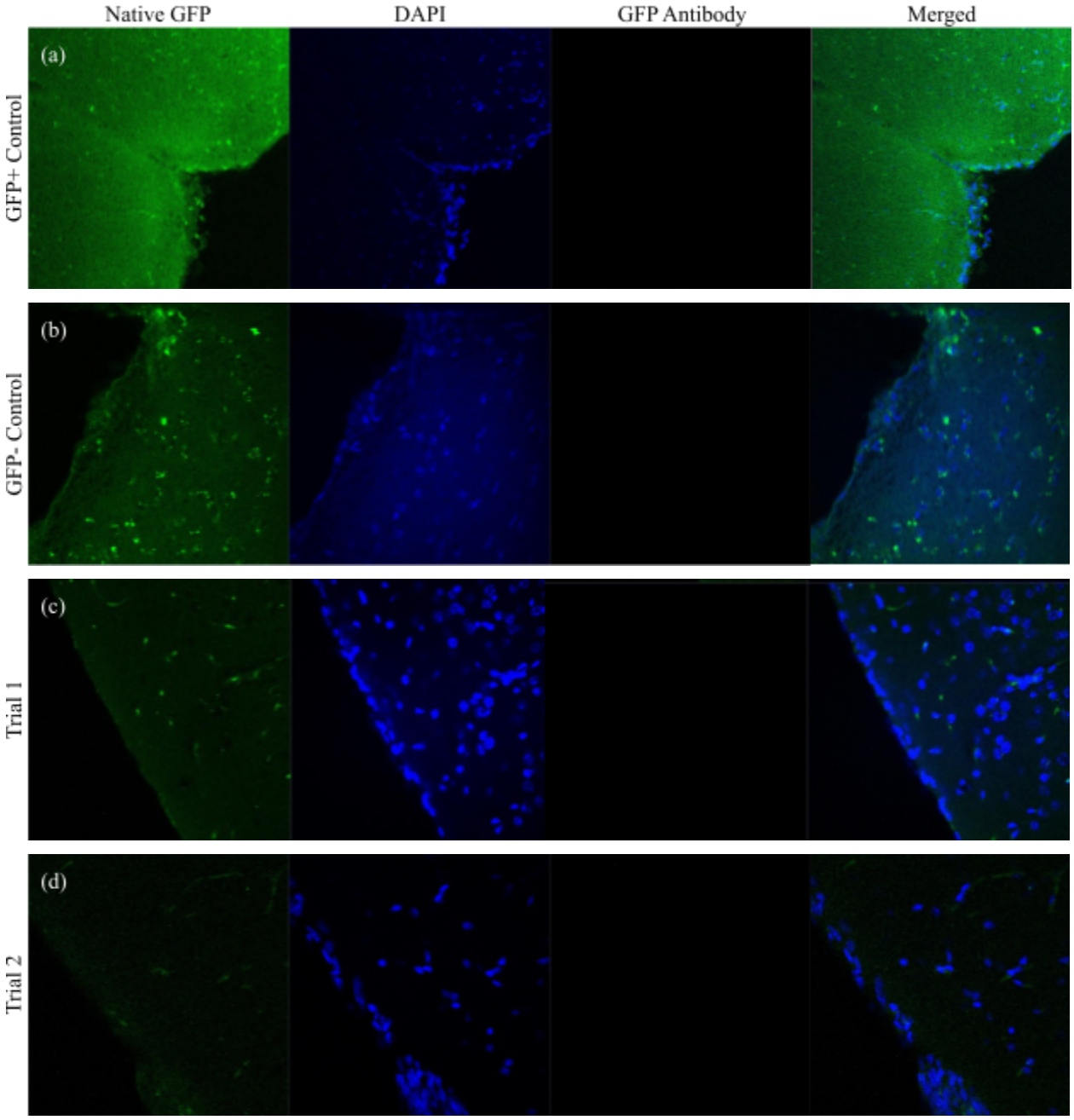

Figure 2. A 480 μm laser was used to view Native GFP fluorescence, and a 355 μm laser was used to view cell nuclei stained with DAPI. A 580 μm laser was used to view GFP antibody fluorescence, and the merged images combine fluorescence from all three lasers. (a-b) The GFP+ control and GFP- control are depicted respectively under each fluorescent condition. (c-d) Two distinct experimental trials are depicted under each fluorescent condition (n=2).

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