

STAT6 Macrophage Polarization in Response to External Fibrotic and Microplastic Stimuli
Through Bioluminescence Temporal Signaturing

Maeve Stiles

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Committee:

Elizabeth Wayne

Cecelia Giachelli

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Department of Bioengineering

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Maeve Stiles

University of Washington

Abstract

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Maeve Stiles

Chair of the Supervisory Committee:

Elizabeth Wayne

Department of Bioengineering

Macrophages are highly plastic immune cells that regulate fibrosis, an aberrant inflammatory response resulting in the excessive buildup of connective tissue. Occurring during organ failure and chronic illness, there is a pressing need to improve the monitoring and prediction tools of immune processes that drive fibrosis. Macrophage based immune cell biosensors releasing a quantifiable bioluminescent output in response to the activation of STAT6, a reporter element linked to anti-inflammatory macrophage phenotype, would allow for temporal and spatially resolved signals to track macrophage states during the inflammatory process. My goal was to optimize a high-throughput, repeatable platform for quantifying STAT6 activation through bioluminescent temporal spectroscopy (BTS). Macrophage STAT6 activation was monitored in incubation with fibroblasts, the connective tissue cells that build up in fibrosis, in two 3D cell-culture platforms, and with nanoplastics present at high rates in

the body. Application of BTS to immune response monitoring will better inform models of fibrosis and inflammation for higher in vivo translation and therapeutic intervention.

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Chapter 1: Introduction to Macrophage Biology in Fibrosis

Summary

This chapter describes the basic knowledge of macrophage origin and function in normal physiological and pathophysiological fibrosis. The importance of dynamic macrophage polarization regulation in maintaining tissue homeostasis is discussed with dysregulation of this transition a hallmark of numerous chronic diseases, namely fibrosis. The role of STAT6 in polarization and in gene reporter cells within macrophages to offer real time signals of gene transcription is highlighted.

Macrophage Origins

Macrophages are innate immune cells that function as tissue sentinels, responsible for identifying and phagocytosing foreign pathogens and toxins, dead cells, and other particulate matter. Macrophages are a widely dispersed organ system, present from birth throughout the lifespan in all vertebrate tissue and organ, constituting one of the most widespread and versatile cell types (Katkar & Ghosh, 2023). They are also crucial in tissue repair and maintenance, serving as key regulators of tissue homeostasis (Murray & Wynn, 2011a; Sanin et al., 2022; Watanabe et al., 2019). Macrophages can be activated by various inflammatory stimuli during an immune response (Arango Duque & Descoteaux, 2014a). As one of the first responders to stress, they respond to danger signals from hormones, neurotransmitters, ligands from DNA, RNA and LPS as

well as cytokines (Hoyer et al., 2019a). Therefore, they are critical in regulating organ-level immunity and responding to cues in their local tissue environment.

Embryo-derived macrophages form tissue-specific resident populations during organogenesis, serving as locally self-renewing regulators for tissue homeostasis (Carrizo et al., 2026; Gordon & Plüddemann, 2017; Lazarov et al., 2023). These tissue-resident macrophages perform tissue-specific regulatory functions, forming a 3D network within each organ where they are identified by tissue-specific gene expression profiles (Mass et al., 2016). Macrophages are involved in complex tissue-specific functions while still able to perform general phagocytotic duties, exhibiting their high plasticity in organ-specific function (Park et al., 2022a). As highly plastic, widespread immune cells, monitoring their specific cell states and spatial functions are critical in assessing their roles throughout the inflammatory response.

Monocytes, derived from bone-marrow precursors, circulate throughout the blood stream and migrate into distinct tissues in the body under homeostatic and inflammatory conditions (Chaudhuri, 2014; H. Li et al., 2018). In homeostatic conditions, monocytes replenish the population of tissue macrophages and dendritic cells (Shi & Pamer, 2011). However, during stress and tissue damage, inflammatory signaling from cytokines, damage-associated molecular patterns (DAMPs) released by dying cells, and pathogen-associated molecular patterns (PAMPs) released by invading organisms result in rapid recruitment of monocytes into tissues (T. A. Wynn & Vannella, 2016a). Once within these tissues, these inflammatory monocytes can differentiate into monocyte-derived macrophages, able to recruit additional macrophages through local and systemic inflammatory signaling (Mildner et al., 2024; Murray & Wynn, 2011b; Park

et al., 2022b; Watanabe et al., 2019). Monocytes recruited from the blood serve to replenish the long-lived tissue-resident macrophage populations. Monocyte-derived macrophages and resident macrophages utilize proliferation as a strategy to control cell numbers in tissue, important to maintaining homeostasis and in mounting injury responses (Epelman et al., 2014). Therefore, distinguishing and responding differentially to inflammatory and homeostatic cues by monocytes and macrophages is critical in forming a regulated, coordinated immune response.

Macrophage Activation through a Complex, Coordinated Process

Macrophages can be activated through a wide range of signals. As the sentinels of the body, their toll-like and scavenger receptors recognize invading ligands first through evolutionarily conserved motifs on microbes and host cells, termed PAMPs and DAMPs, respectively (Greene et al., 2022). Pathogen-associated molecular patterns (PAMPs) activate specific pattern recognition receptors on macrophages to trigger intracellular signaling pathways. These pathways induce the expression of a host of proteins and enzymes, resulting in the production of a variety of cytokines and chemokines.

Molecules exuded from injured or stressed cells can activate macrophages through danger-associated molecular patterns (DAMPs) through their phagocytic functions (Stefater et al., 2011). Macrophages can process and display various antigens with the major histocompatibility complex (MHC) class II molecules, which are recognized by T helper cells. These cells can activate B cell lymphocytes and macrophages to mount an immune response (Roche & Furuta, 2015). Furthermore, activation of macrophages can occur directly from cytokines, small proteins that serve as communication mediators between immune and non-immune cells that are responsible for regulating

inflammation. Each cytokine is produced by a specific cell population, typically lymphocytes and macrophages, in response to a different stimulus, binding to a specific cell surface receptor. These cytokines influence cell function and regulate how macrophages mount their immune response (Arango Duque & Descoteaux, 2014b; Mosser & Edwards, 2008). Therefore, macrophage phenotypic expression and activity arise from a complex, coordinated process of interacting elements in the cellular environment. As macrophage behavior is influenced largely by microenvironmental signaling, changes to the cellular environment have large implications in the mounting immune response.

Macrophage Polarization

Depending on different microenvironmental cues and signals, macrophages can develop distinct phenotypes with diverse functions (Lawrence & Natoli, 2011a; H. Li et al., 2018). These diverse signals can bias macrophages phenotypes toward “classically activated” (M1) or “alternatively activated” (M2) macrophages (Arango Duque & Descoteaux, 2014a). The development of these specific phenotypes is termed “polarization” and is triggered by signals from apoptotic cells, hormones, cytokines, or immune cells. M2 and M1 polarized macrophages fulfill distinct functions due to microenvironmental cues. Specifically, M2 macrophages have been shown to promote tumor growth while M1 macrophages can provide both tumor-promoting and anti-tumor properties (Strizova et al., 2023). Existing along a spectrum of activation, understanding and responding to local cues is important in mounting a context-specific immune response.

M1 macrophages are more commonly associated with pro-inflammatory responses, therefore resulting in more anti-tumor effects. Their polarization is typically triggered by Th1 cytokines, such as IFN- γ , TNF α or by bacterial lipopolysaccharide (LPS) recognition (Shapouri-Moghaddam et al., 2018). These inflammatory M1 macrophages release pro-inflammatory cytokines such as TNF α , IL-1, IL-6, IL-12, IL-23, Type 1 IFN- γ , CXCL1-3, CXCL-5 and CXCL8-10 (Arango Duque & Descoteaux, 2014b; Geissmann et al., 2010; N. Wang et al., 2014). They are also responsible for the production of reactive oxygen species (ROS) that engage in pathogen killing and tissue damage. M1 macrophages are associated with increased microbicidal activity, high antigen presentation due to elevated MHC class II expression and increased endocytic function and killing of intracellular pathogen (Lawrence & Natoli, 2011b; Martinez, 2008). Specifically in cancer and other pathologic conditions, they are responsible for eliciting antibody-dependent cellular phagocytosis (Strizova et al., 2023). Overall, the role of M1 macrophages in inflammation has been largely explored, tasked with destroying pathogens and supporting anti-tumor progression.

M2 macrophages operate on an oppositional functional spectrum from M1 macrophages. They are involved in tissue restoration, parasitic response, immune regulation and the dampening of inflammatory signals (Strizova et al., 2023). Due to the wide roles these M2 macrophages play, they are further divided into subtypes M2a, M2b, M2c, and M2d, each of which display unique markers and are polarized in response to different molecules and environments (De Paoli et al., 2014; Shapouri-Moghaddam et al., 2018). The Th2 cytokines IL-4 and IL-13 induce an M2a macrophage phenotype, which express high levels of the ECM protein fibronectin that

result in wound healing and fibrotic lesion formation (P. Li et al., 2022; Shapouri-Moghaddam et al., 2018). Their high expression levels of the mannose receptor CD206 allows them to phagocytose dead cells and debris (Anders & Ryu, 2011). Type 2 macrophages (M2b) result from stimulation with IL1 β or LPS through Toll-like receptors and immune complexes and are characterized by low IL-12 and high IL-10 production, which induces an anti-inflammatory immune response (Martinez, 2008). M2c macrophages result from stimulation with IL-10, TGF β or glucocorticoids, displaying high expression of CD163, involved in the clearance of haptoglobin/ hemoglobin complexes (Strizova et al., 2023). These “deactivated” macrophages are engaged in the end-stages of immune reactions and inflammatory resolution, as they downregulate pro-inflammatory cytokines, increase debris scavenger activity and continue the pro healing paradigm (X. Huang et al., 2018; Martinez, 2008). Lastly, M2d macrophages, activated by adenosine receptor agonist stimulation and IL6, are associated with tissue repair and cancer cell invasion in vitro (X. Huang et al., 2018; Nikovics et al., 2020). The breadth of functional roles of M1/M2 macrophages and their polarizability in disease and inflammation makes monitoring of macrophage cell state crucial in treatment interventions. Further, understanding when these phenotypic macrophage polarization occurs is crucial towards understanding the disease/inflammatory signaling progression. Macrophage polarization is a central process in immune regulation, with transitions between the pro-inflammatory M1 phenotype to the late reparative M2 phenotype at key times in wound repair critical for maintaining tissue homeostasis. Crucially, macrophages polarization exists as a spectrum, composed of different properties and phenotypic elements with M1 and M2 positioned as opposing elements of this scale.

Therefore, a stimulus-dependent activation network with specific macrophage phenotypes identified by their activation agent, such as IL4, and the pathway this activates is a clearer way to identify and describe this coordinated environmental shift (Murray et al., 2014). The dysregulation of this complex, dynamic shift is involved in chronic diseases, such as atherosclerosis, autoimmune diseases such as arthritis, lupus and immune thrombocytopenia, and fibrosis (Horn & Tacke, 2024; J. Li et al., 2023; J. Wang et al., 2024; Wu et al., 2025). Overall, macrophages, operating on the front lines of the innate immune system, must maintain the balance between destructive, pro-inflammatory signaling responses and wound-healing, anti-inflammatory responses.

Macrophages in Fibrosis

Fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) resulting from sustained tissue dysfunction and proliferation of fibroblasts (Y. Jiang et al., 2024a). Fibrosis has been associated with up to 45% of fatalities, largely due to its association with organ failure and malfunction during chronic illness or after organ transplants resulting in dysregulated tissue remodeling (Antar et al., 2023). While a result of diverse factors and affecting nearly every organ in the body, fibrosis shares common underlying mechanisms such as an overactive inflammatory response, high fibroblast activation and dysregulated tissue remodeling following injury repetition (Ku et al., 2024). Fibrosis stems from persistent inflammatory conditions rooted in lymphocyte-monocyte interactions that lead to continued secretion of pro-fibrotic cytokines, growth factors and enzymes (T. A. Wynn, 2004). This can result in impaired organ structure and function with tissue-specific symptoms related to excessive invasion of inflammatory cells. Long though to be irreversible, clinical, preclinical and mechanistic

studies have revealed fibrosis to be a largely dynamic process, a part of a complex pathway from tissue damage to inflammation that can be targeted with potential therapeutics along crucial points of this process.

During steady state, tissue-resident macrophages are comprised of both embryonic and monocyte derived populations of tissue-resident macrophages whose functions are determined by their tissue environment (Hoyer et al., 2019b). In response to environmental cues including cytokines, growth factors and molecular patterns, macrophages undergo dynamic phenotypic transitions between different functional states. Their phenotypic gene expressions can switch between pro-inflammatory M1 phenotypes to M2 anti-inflammatory gene signatures (Lawrence & Natoli, 2011a). If an inflammatory response is resolved quickly, normal tissue function and structure is restored. However, prolonged or dysregulated inflammatory signaling, characteristic of disease states, can lead to the development of fibrosis, limiting tissue function and eventually leading to organ failure and death (T. A. Wynn & Vannella, 2016b).

Fibrosis is also the end result of a the foreign body response (FBR), an inflammatory response resulting from implants, which begins as an acute inflammatory response and leads to fibrotic tissue formation around the foreign material (Dickenson et al., 2026). Key to this shift is the transition of macrophages from a pro-inflammatory to an anti-inflammatory state, where the buildup of M2 macrophages that recruit fibroblasts to the foreign material surface leads to the development and thickening of this fibrotic capsule. While mitigation of the FBR has ranged from changing biomaterials surface modifications, incorporating drugs, or other changes, nothing has been able to ameliorate the dynamic FBR tissue-specific effects. Therefore, new technologies are

crucial towards understanding when and how this macrophage transition occurs to prevent the FBR and increase knowledge of pivotal times and spaces to incorporate drug and treatment paradigms.

Crucially, macrophages and the pro-fibrotic mediators and chemokines they secrete are involved in all stages of the fibrotic process, making them the master “regulators” of fibrosis (T. Wynn & Barron, 2010). As the polarization status of macrophages is highly plastic, dependent on environmental signals and exposure to stimulation agents, they are highly affected by changes in disease states and injury. Therefore, the transition between anti- and pro-inflammatory gene states along the course of disease, specifically fibrosis related to an aberrant inflammatory phenotype, is important in maintaining tissue homeostasis or pivoting to a fibrotic phenotypic state.

Following injury, macrophages are polarized into an M1 phenotype, mounting a pro-inflammatory response as the first stages of wound healing. As the inflammatory response continues, they transform into M2 macrophages, responsible for the production of the anti-inflammatory cytokines IL-10 and TGF β in addition to increasing phagocytotic activity that prevents the formation of cellular granulation and tissue scars (Y. Jiang et al., 2022; Shapouri-Moghaddam et al., 2018). In addition, anti-inflammatory mediators IL-10 and TGF β 1 secreted from M2 macrophages alongside M2 cell surface receptors PD-L1 and PD-L2 suppress the immune system and temper inflammation. Altogether, this promotes tissue repair and wound healing through the secretion of growth factors that lead to cellular proliferation and blood vessel growth as well as recruiting tissue fibroblasts to differentiate into myofibroblasts (Murray & Wynn, 2011b). During normal wound healing, granulation tissue disappears once replication and the

movement of epithelial cells, epithelialization, has been completed through the apoptosis of fibroblasts, myofibroblasts and vascular cells (Desmoulière et al., 1995). In fibrosis, this apoptosis is lacking, leading to a sustained presence of connective tissue containing fibroblasts, ECM, specifically collagen, and small vessels (Gabbiani, 2003).

The TGF- β cytokine is a well-studied regulator of the ECM, with the production of its isoform TGF- β 1 linked to tissue fibrosis. Chemokines such as CCL3 work with these pro-fibrotic cytokines to recruit macrophages to tissue damage sites (T. A. Wynn, 2004).

As M2 macrophages are a major source of both, they are crucially intertwined with fibrosis. In addition, the type 2 cytokines IL-4, IL-5 and IL-13 are all linked to fibrosis, further linking aberrant anti-inflammatory M2 macrophages to pathological fibrotic development (Chiaramonte et al., 1999; Ong et al., 1998; Reiman et al., 2006).

Specifically, sustained recruitment and activation of macrophages polarized by IL4, or M2a macrophages, have been suggested to play a role in fibrosis (T. A. Wynn & Ramalingam, 2012a). This is further proven by the formation of fibrotic scars by specifically M2a macrophages through TGF β 1 during cerebral ischemia (J.-G. Huang et al., 2023). However, while M2 macrophages are suggested to play a role in fibrosis, the temporal and pathway specific activation of fibroblasts remains unknown. Necessary in every step of the tissue repair process, macrophage differentiation and polarization at crucial time points are vital for physiological, healthy tissue function and wound repair. Any changes in signaling or environmental contexts can lead to uncontrolled cytokine, chemokine, or growth factor production or deficient inhibitory macrophage generation that can lead to fibrosis.

In addition to producing pro-fibrotic cytokines and chemokines, inflammatory macrophages sustain and activate the production of myofibroblasts, the cells responsible for ECM matrix production (Vannella & Wynn, 2017). In a two cell circuit between macrophages and myofibroblasts, myofibroblasts secrete colony-stimulating factor (CSF) for macrophage localization, while both macrophages and myofibroblasts themselves secrete platelet-derived growth factor (PDGF) for myofibroblasts (M. Adler et al., 2020; Bonner, 2004). Therefore, the crosstalk of pro-fibrotic macrophages and fibroblasts are critical in the creation of scar tissue and pathological ECM deposition.

Overall, while M1 macrophages induced by type 1 cytokines such as IFN- γ are shown to have antifibrotic activity, M2 macrophages activated by type 2 cytokines such as IL-4, IL-5 and IL-13 have been found to initiate the fibrotic phenotype (Oldroyd et al., 1999; T. A. Wynn, 2004; T. A. Wynn et al., 1995). Therefore, a better understanding of this M2 activation pathway resulting in the release of profibrotic factors is crucial in understanding fibrosis inflammatory progression and finding potential targets during crucial stages of this fibrotic pathway.

STAT6 Signaling as a Measure of Macrophage Polarization and Role in Fibrosis

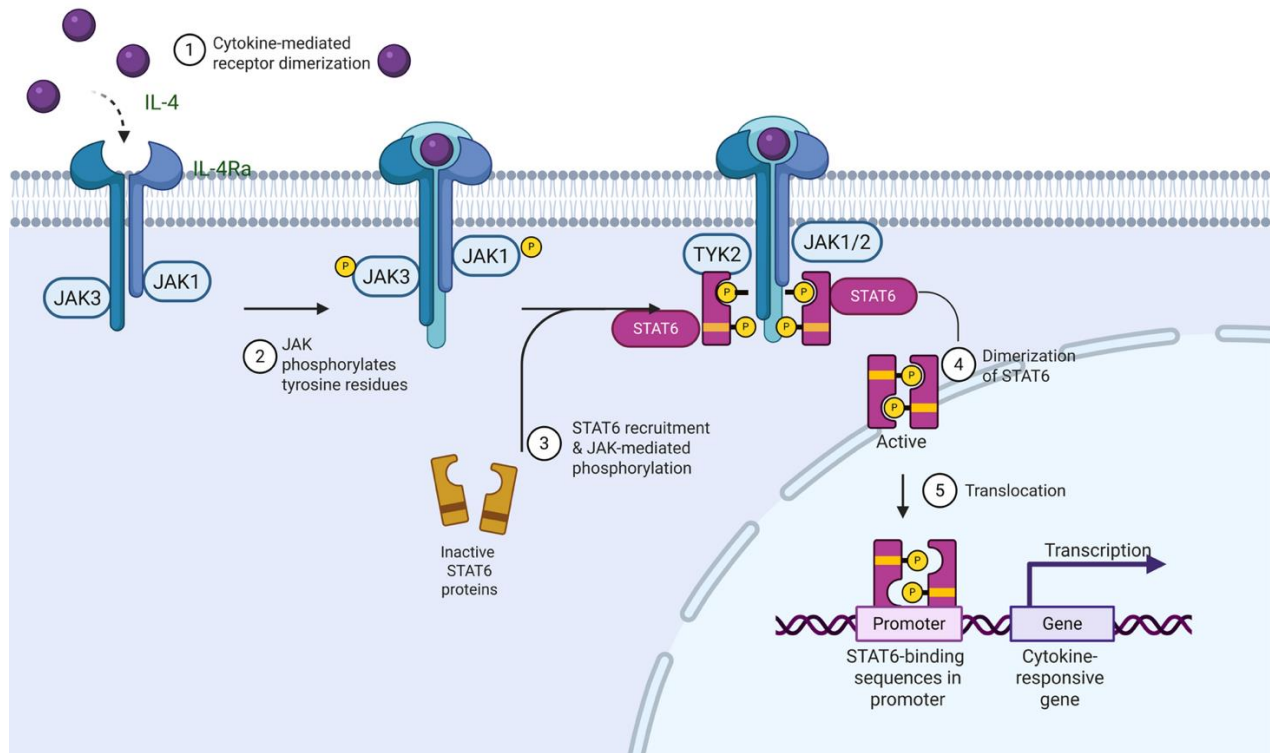


Figure 1 Activation of JAK/STAT6 Pathway by IL4

Note: The STAT6 Pathway by Zhang, Shuni, Shuzhen Xiong, Xinyang Liu, Jiajia Cao, Ningning Yue, and Chongyang Wu. "STAT6: Multidimensional Analysis from Hematopoietic Immune Regulation to Pathogenesis Mechanisms and Therapeutic Targets in Hematologic Malignancies." Annals of Hematology 105, no. 4 (2026): 183. <https://doi.org/10.1007/s00277-026-06932-2>. Adapted with Permission.

STAT6 is a signal transducer and transcription activator abundant in many tissues and organs that is essential for driving M2 activation (Fu et al., 2019; Lawrence & Natoli, 2011a). STAT6 has been shown to be a major regulator of macrophage anti-inflammatory polarization, activated through the two cytokines IL-4 and IL13 (Goenka & Kaplan, 2011; Takeda et al., 1996). Both cytokines mediate the type 2 immune responses, which when activated for prolonged periods can lead to fibrosis (Gieseck et al., 2018). However, mouse macrophages and human and mouse monocytes showed

greater sensitivity to IL-4, making them a more effective agent in STAT6 activation and macrophage M2 polarization (Junttila et al., 2008). STAT6 specifically regulates M2 macrophage polarization, shown to have antagonistic effects with STAT1, responsible for gene expression of M1 associated phenotypic changes (Lawrence & Natoli, 2011b). Therefore, STAT1, crucial for M1 activation while STAT6 is selectively involved in M2 polarization, highlighting its value as a M2 specific gene signature for selective polarization paradigms.

Once IL4 binds to its receptor, tyrosine residues are phosphorylated by activated Janus Kinases (Jak), allowing STAT6 to bind onto this receptor through its SH2 domain. Upon STAT6 docking, Jaks phosphorylates the tyrosine-641 on STAT6, facilitating its formation of a homodimer and translocation to the nucleus to regulate transcription (Goenka & Kaplan, 2011; Mikita et al., 1996; Quelle et al., 1995). This is depicted in **(Figure 1)**. In macrophage cultures, STAT6 has been shown to bind to macrophages within 30 to 60 minutes of IL4 exposure, highlighting its rapid activation (Caratti et al., 2023; Ostuni et al., 2013).

STAT6 plays a large role in regulating the allergic response and immune response. Crucially, STAT6 knockout in mice was found to inhibit ECM protein expression, whose overexpression is a hallmark of fibrosis, and suppressed kidney fibrosis through interaction with PPAR γ and its fatty acid oxidation (FAO) related genes (J. Li et al., 2022). STAT6 is implicated in cardiac fibrosis, as its activity was increased surrounding injured hearts, producing larger amounts of β 1-adrenic receptor (β 1-AR) and inducing inflammatory signaling pathways (Zhang et al., 2020). Further, STAT6 is associated with TAM polarization through IL4 as a mediator, highlighting its role in specifically M2

polarization rather than the differential activation of the M1 phenotype. While both are macrophage subsets, M1 and M2 activation occur as a result of opposing stimulatory pathways, allowing for selective M2 activation without the risk of M1 activation, if appropriate stimulation agents are utilized.

Overall, STAT6 is a well-defined transcription factor known to be rapidly activated by IL4 to polarize macrophages into an anti-inflammatory state (**Figure 1**). Furthermore, it has been associated with both renal and cardiac fibrosis, along with ECM proliferation, correlating STAT6 activation with fibrotic phenotypes. Further, it is implicated in a host of diseases and responses, such as the FBR, suggesting that STAT6 activation serves as both a proxy for macrophage polarization and important in fibrotic disease intervention. Overall, STAT6 activation can be utilized to track macrophage polarization and the development of fibrosis, as prolonged M2 macrophage activation is implicated in fibrosis.

Reporter Cell Lines for Real-Time Gene Monitoring

Reporter cell lines can produce a signal in response to recognition of a specific cues using fine-tuned gene expression. Briefly, the reporter gene is placed downstream of a promoter or other regulatory element to drive gene expression when a signal is present, resulting in a detectable signal through expression of an observable reporter gene.

Typical reporter genes include fluorescent proteins like GFP and RFP or luciferase, an enzyme that controls bioluminescence (Yi et al., 2025a). Therefore, following excitation, in the cases of fluorescent proteins, or oxidation, in the case of luciferase, a quantifiable signal can be tied to gene activity. Reporter cell lines are largely utilized in drug

discovery, gene expression regulation, signal transduction tracking, receptor-ligand interactions, and protein localization and translation (Yi et al., 2025b). Therefore, resolution of a quantifiable signal output upon exposure to a cue can inform cellular and molecular processes in vitro, which is key in fibrosis formation and progression.

The choice of a reporter gene is crucial towards tracking reliable signal output, as the signal output is what is used to track the activation of a particular pathway or biological process. Fluorescence molecules for bioimaging probes are limited by photostability, seen in genetically encoded fluorescence proteins, and low selectivity to target molecules, seen with fluorescent dyes and nanoparticles (Ozawa et al., 2013).

Therefore, their applications in accurately tracking transcriptional changes on a time resolved scale is limited. Compared to other imaging modalities including fluorescence based reporter systems, bioluminescence imaging and analysis produce higher signal to noise (S/N) ratios with lower background noise, a broader dynamic signal range and molecular design versatility (Ozawa et al., 2013). The use of different luciferases derived from new bioluminescent organisms that catalyze reactions with their substrates luciferins have improved optical qualities, generating novel enzyme-substrate pairs with higher specific activity, lifetime, stability, and higher reporter quantification at lower expression levels (Hall et al., 2012). Therefore, reporter cell lines with bioluminescence-based reporter genes allow for a higher signal intensity and readable output, facilitating the monitoring of gene expression in real time.

Utilizing Macrophage Plasticity in Disease Contexts

Macrophages are highly plastic immune cells that play a role in development, homeostasis, and repair and regeneration through their response to a wide range of diverse environmental cues. Present across all tissues, they adapt their functional phenotype in a complex, coordinated process due to environmental cues and signals. As macrophages are polarized along a spectrum, with M1 and M2 phenotypes operating on opposing ends, understanding the phenotypic and temporal shift that occurs along this transitional spectrum is important in understanding what dynamic processes macrophages are inducing in response to disease states and inflammatory pathways. Crucially, a lack of real time, temporal and spatial resolved cues of the fibrotic inflammatory process, with macrophages as key effector cells, limits therapeutic intervention. As fibrosis is associated with macrophage, IL-4 derived activation along the STAT6 signaling pathway, the quantification of these polarized cells would help to when and how immune responses and inflammation associated with fibrosis occurs.

Chapter 2: Bioluminescence Measurement Techniques to Monitor Spatial and Temporal Macrophage States

Summary

This section describes the basic concepts behind temporally and spatially resolved bioluminescence temporal spectroscopy (BTS) measurements to be utilized for tracking macrophage polarization in fibrosis. Following the establishment of basic conditions, various substrate concentrations and activating cytokine media were tested in an initial BTS experimental workflow in order to optimize bioluminescent read outs for monitoring RAW 264.7 STAT6 macrophage activation to be utilized for future experimental conditions.

Bioluminescence Temporal Spectroscopy (BTS) to Monitor Spatio-Temporal Macrophage States

As highly plastic, reactive immune cells, macrophages respond quickly to environmental cues. Therefore, knowledge of the dynamic, anti-inflammatory properties of macrophages over the course of fibrosis, a disease characterized by widespread chronic inflammation, could be key in harnessing macrophages wound healing and repair functions to prevent pro-fibrotic phenotypes and for other therapeutic aims. The use of engineered bioluminescent viruses through bioluminescence imaging serves to noninvasively measure the real-time viral signatures in cells (Smith et al., 2025).

Further, Bioluminescence Imaging (BLI) has emerged as a form of semiquantitative measurement due to the correlation between cell number and bioluminescent signal, allowing for visualization of biological processes (de Almeida et al., 2011). The use of BLI has allowed for the use of Bioluminescence Temporal Spectroscopy (BTS) to dynamically measure and quantify macrophage polarization.

As macrophages/monocytes are highly plastic, dynamic cell populations that are continuously reacting to and evolving alongside microenvironmental signals, tracking responses to environmental cues over long periods of time in real time is crucial towards understanding the full nature of macrophage interaction with the microenvironment. Current data surrounding the physiological, systematic responses of macrophages to changing environments in the context of disease is lacking due to traditional endpoint analysis of macrophage gene signatures (Lawrence & Natoli, 2011a). Differentiation and polarization, crucial for macrophage characterization, are measured through flow cytometry, RNA sequencing and proteomics (Misharin et al., 2013; Stark et al., 2019). While these phenotyping techniques have uncovered crucial omics information regarding macrophage function, challenges exist in their application towards uncovering macrophage behavior. Difficulties in balancing proteome coverage and quantitative reliability, lack of spatial context and dynamic signaling, low sensitivity to lower abundance markers, ill-defined macrophage subset markers, and lack of long-term response monitoring have painted an incomplete understanding of macrophage response to their changing environments (Italiani & Boraschi, 2014; Martinez & Gordon, 2014; Molla Desta & Birhanu, 2025; Schoof et al., 2021). Further, end point analysis limits the resolution of dynamic, real-time macrophage polarization states that are

crucial in monitoring disease and fibrotic progression. Overall, current data surrounding macrophage roles in fibrosis are incomplete, due to limitations in current tools to monitor fibrosis progression. Therefore, the application of BTS to macrophage involvement in the fibrotic microenvironment is crucial in progressing this field of study.

Luciferase-Luciferin Pair for BTS

Bioluminescence temporal spectroscopy (BTS) serves as way to quantitatively measure real-time changes in macrophage polarization states. The technique BTS is founded on the chemical process of bioluminescence, resulting in the emission of visible light through the oxidation of the substrate luciferin by the protein luciferase (Kaskova et al., 2016). As bioluminescence does not require external light sources, complications associated with fluorescence such as light scattering or background interference are removed, improving sensitivity, spatial resolution and widening dynamic ranges for measuring quantitative signal intensities.

Luciferases are a family of photoproteins isolated from different bioluminescent organisms (Ozawa et al., 2013). These enzymes can generate light through the oxidation of their complimentary luciferin, photon-emitting substrate (Hall et al., 2012). Traditional bioluminescent systems have relied on D-luciferin, paired with the popular Firefly luciferase enzyme, both of which were the first to be discovered. With the increasing use of bioluminescent systems to facilitate real time monitoring of physiological processes, artificially engineered bioluminescent platforms have emerged to maximize signal intensity and stability (Kaskova et al., 2016). The development of

these novel bioluminescence systems involves engineering both an enzyme and substrate in concert.

Recently developed in 2012 by Hall and colleagues, NanoLuc (nLuc) is a small, highly stable and soluble novel luminescent protein engineered from a deep-sea shrimp luciferase (Hall et al., 2012; Y. Su et al., 2020). Its optimized novel substrate furimazine produces a luminescent signature with a half-life above 2 hours, with higher specificity than previous luciferases (Boute et al., 2016). In combination, this new system has prompted ~2.5-million-fold improved luminescence in mammalian cells with a ~150-fold greater specific activity than traditional firefly luciferase (Hall et al., 2012). Further, its small 19 kDa size and enhanced luminescence makes it ideal as a protein fusion tag with other genes such as STAT6, to explore the dynamic macrophage polarization dynamics.

The nLuc substrate furimazine has limited depth detection sensitivity due to poor solubility in water and its blue light emission which makes deep tissue imaging difficult. Further, its potential cytotoxicity has limited its concentration of administration to 20 μ G per day (Smith et al., 2025; Y. Su et al., 2020). This has spurred exploration into other nLuc-based bioluminescent reporters that are more water-soluble than the substrate furimazine. Su et al developed the substrate fluorofurimazine (FFz) synthesized from the original furimazine substrate that has a 1.2 to 2.93 higher relative flux than furimazine due to its increased bioavailability and water solubility (Smith et al., 2025). Crucially, it has been shown to be optimal for imaging distributions of immune cells where its intensity of light production allows for high sensitivity (Y. Su et al., 2020). The NanoLuc/FFz pair were also shown to provide an 8.6-fold higher brightness when

compared to the NanoLuc/furimazine pair (Gaspar et al., 2021). Therefore, the combination of fluorescent donor, nLuc, with FFz were utilized as the luciferase-luciferin pair for monitoring macrophage dynamics.

Response Element Reporters

To track macrophage polarization in vivo, the reporter gene (luciferase) must be expressed by the imaged cells, necessitating the delivery of the genetically encoded imaging reporter into the cells. The reporter gene and probes must be optimized to study long term cellular activity at a high sensitivity level while not altering the polarization activity itself (de Almeida et al., 2011). NanoLuc (nLuc) has been developed and validated as a luciferase reporter gene, able to be easily incorporated into the viral genome without the ATP cofactor, allowing its extracellular detection and quantification (Smith et al., 2025). Coupling gene expression with an nLuc luciferase reporter gene is a simple and effective platform for measuring dynamic gene expression changes with bioluminescence imaging (BLI), enabling long term tracking.

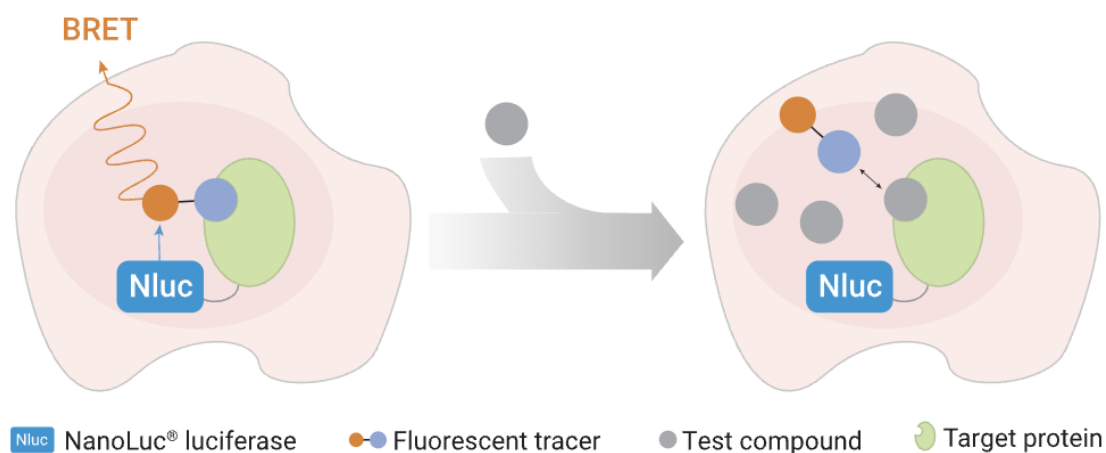


Figure 2 NanoBRET Target Engagement Assay Principle

The target protein expressed as the NanoLuc luciferase fusion is bound by the NanoBRET fluorescent tracer on its own fusion protein, resulting in energy transfer and BRET signal when the two are brought into proximity (Adapted from NanoLuc Luciferase. Promega. <https://www.promega.com/resources/technologies/nanoluc-luciferase-enzyme/>)

Bioluminescence resonance energy transfer (BRET) involves the energy transfer between light emitting and light accepting molecules, allowing for greater penetration depth in bioluminescent signal and activation. Specifically, it couples fluorescent luciferases as energy donors with acceptors being fluorescent probes or proteins to create direct fusion luciferase-fluorescent protein moieties (Schaub et al., 2015; Smith et al., 2025). NanoLuc-based BRET probes were designed to monitor macrophage polarization, composed of NanoLuc, the bioluminescent donor luciferase, fused to fluorescent proteins, enhanced Nano-Lanterns (eNLs) (Suzuki et al., 2016; Tedeschi et al., 2024). Briefly, NanoLuc, the donor luciferase, and fluorophore acceptor are bound to proteins of interest. When these proteins interact with the appropriate stimuli present, the luciferase and fluorophore are brought into proximity, causing an energy transfer from the luciferase to the fluorophore in a BRET reaction (Tang et al., 2019) (**Figure 2**). Five eNLs have been developed, all of which demonstrated a higher luminescence

intensity than nLuc from 4 to 1.8-fold across the color variants CeNL, GeNL, YeNL, OeNL and ReNL. Therefore, selection of either of these eNLs would produce a bright, reliable signal to ensure maximal bioluminescent output when paired with a gene-specific promoter.

The JAK-STAT6 signaling pathway drives anti-inflammatory polarization associated with wound healing upon activation with IL-4, therefore serving as a proxy for M2a macrophage polarization (Andrews et al., 2002; Dorrington & Fraser, 2019). Therefore, STAT6 serves as a valuable promoter for tracking the dynamics of M2a macrophage polarization through IL-4-mediated activation. Engineered STAT6-CeNL reporters were introduced into RAW264.7 cells, a model macrophage cell line, via viral transduction. Before use, the cell lines with the BRET reporter expression were validated by M2 polarization. A basic overview of the STAT6CeNL reporter structure within macrophages and activated upon IL4 exposure is depicted in **Figure 3**, with the BRET reporter elements included in its design. Overall, the nLuc BRET platform serves as a valuable way to quantify STAT6 mediated signaling in macrophages, providing a platform for monitoring temporally resolved signaling in macrophages.

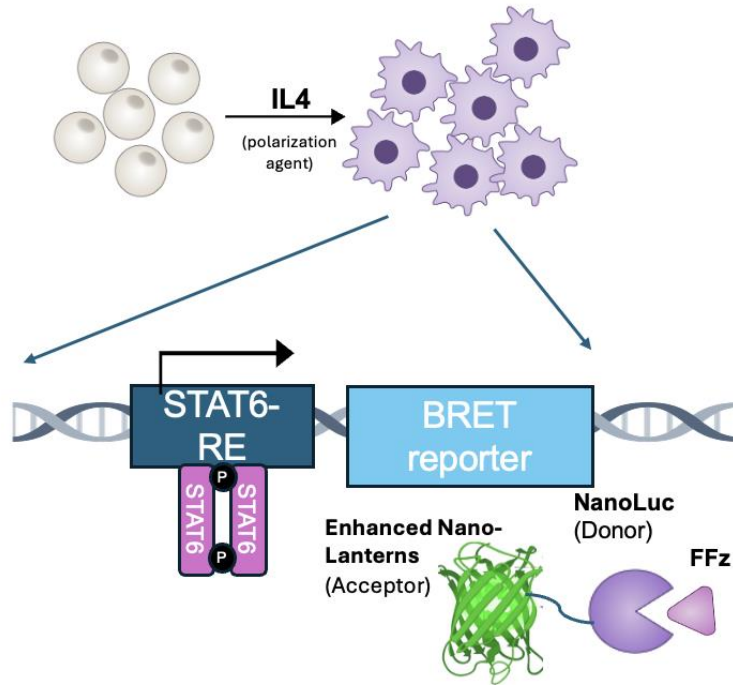


Figure 3 IL4 Mediated Macrophage STAT6 Bioluminescence Based on a BRET Reporter System

Upon IL4 stimulation, the STAT6 signaling pathway is activated, leading to transcription of STAT6 upstream of a BRET reporter that results in a bioluminescent signal when the acceptor, the enhanced nanolanthorn, and the donor, NanoLuc, are brought into proximity with each other in the presence of the substrate FFz. *Made with Biorender.*

Titration of Luciferin Substrates

At higher nLuc concentrations, the substrate has become limiting, resulting in missing bioluminescent signal and an underestimation of gene activity, crucial for understanding the complex signaling dynamics in macrophage polarization (Boute et al., 2016).

Therefore, titrations to determine the optimal concentration, timing, and length of the FFz substrate that balances optimal bioluminescence activity with the mitigation of cell death is necessary to fully monitor macrophage polarization for in vivo and in vitro contexts.

I wanted to optimize an FFz dosing regime for maximizing cell bioluminescent signatures when paired with the luciferase NanoLuc while minimizing cell death.

Therefore, I selected a range of FFz and IL4 concentrations according to previous literature (Smith et al., 2025). I selected IL4 concentrations that ranged between 1-100 ng/mL in accordance with literature that suggested a range between 20 ng/mL and 40 ng/mL had the clearest effect on macrophage phenotype (Pajarinen et al., 2015). Therefore, I wanted a range of IL4 concentrations that targeted below and above this range to confirm its optimal dosage regime in a bioluminescent based experimental design.

Determination of Optimal FFz Concentration

While determination of an optimal luciferin-luciferase pair is crucial to bioluminescence platforms, optimizing the concentration of substrate is necessary for ensuring the balance of substrate saturation with potential cytotoxicity. Therefore, determining the optimal concentration administration of FFz is important towards understanding the full intensity of bioluminescent activity and therefore quantification of biological processes. Previous literature had found FFz at 0.0075 mM, 0.015 mM and 0.0375 mM showed a higher luciferase activity with less cytotoxicity and a better signal to noise ratios than a concentration of 0.05 mM furimazine (Smith et al., 2025). Compared to 0.05 mM furimazine, 0.0375 mM FFz showed improved toxicity, a higher and more sensitive flux signal, and a higher signal to noise ratio than furimazine. Both 0.015 mM and 0.0375 mM FFz maintained over 95% flux intensity for up to 7 minutes, with 0.0075 mM showing a delayed signal intensity, but with a higher long term signal intensity (Smith et al., 2025). Taken together, this suggests that FFz is a better substrate than furimazine, with the optimal FFz concentration between 0.015 and 0.0075 mM. Other literature has used concentrations of 10 μ M FFz to measure NanoLuc cell bioluminescent activity, but

without comparison to other FFz concentrations (Gaspar et al., 2021). Altogether, much work has focused on the benefits of FFz as a substrate for the NanoLuc luciferase without exploring the optimal concentration for BLI measurements (Praditya et al., 2025).

I hypothesized that a lower concentration of FFz was necessary to produce a reliable bioluminescent signal than what was used in previous studies. Therefore, I tested four different concentrations of FFz to determine the most effective dose that balanced maximal signal output with minimal cell toxicity on macrophage engineered reporter cell lines. FFz was tested at concentrations of 10 μ M, 25 μ M, 50 μ M and 100 μ M with macrophages polarized using four different concentrations of IL4 (1 ng/mL, 20 ng/mL, 40 ng/mL and 100 ng/mL) in addition to a control group consisting of no IL4 activation. IL4 was used to activate the STAT6 response element at varying concentrations to ensure maximal activation of the macrophage M2a BRET gene reporter constructs for monitoring macrophage polarization. RAW 264.7 STAT6 macrophages were seeded in a 96 well plate in 100 μ L DMEM media at 30,000 cells/well and allowed to rest for one day. At Day 0, respective IL4 and FFz concentrations were added into the 96 well plate in 100 μ L DMEM media and bioluminescence signals were immediately taken with the Byonoy. Bioluminescent signals were measured in the multimode microplate reader (Spark) at 24-hour interval time points over the course of 72 hours after initial macrophage polarization. FFz was supplemented before each microplate reading with the respective concentration (either 10, 25, 50 or 100 μ M) (**Figure 4A**).

Looking at Figure 4B-E, bulk luminescence measurements across 72 hours showed sharp, consistent increases in bioluminescent intensity of cells dosed with 10 μ M FFz,

independent of IL4 polarization concentration. Notably, signal intensity with 10 μ M FFz increased the most from day 24 to 48 hours across all IL4 concentrations, and continued to rise at 72 hours, the conclusion of the experiment (**Figure 4B**). For the highest FFz concentration, 50 μ M and 100 μ M, there was an immediate, sharp decline in bioluminescent signal intensity that continued from day 0 to day 3 for all IL4 concentrations. In addition, signal intensity, signifying both macrophage polarization and cell reporter presence, was 10-fold lower across days 1-3 for the 50 and 100 μ M concentrations (**Figure 4D,E**). As similar IL4 dosing regimens in 10 μ M and 25 μ M FFz conditions were used, this is more likely due to increased cell death and potential cytotoxicity with higher FFz concentrations than to a lack of M2 polarization, especially since IL4 is a known activator of the M2 anti-inflammatory pathway.

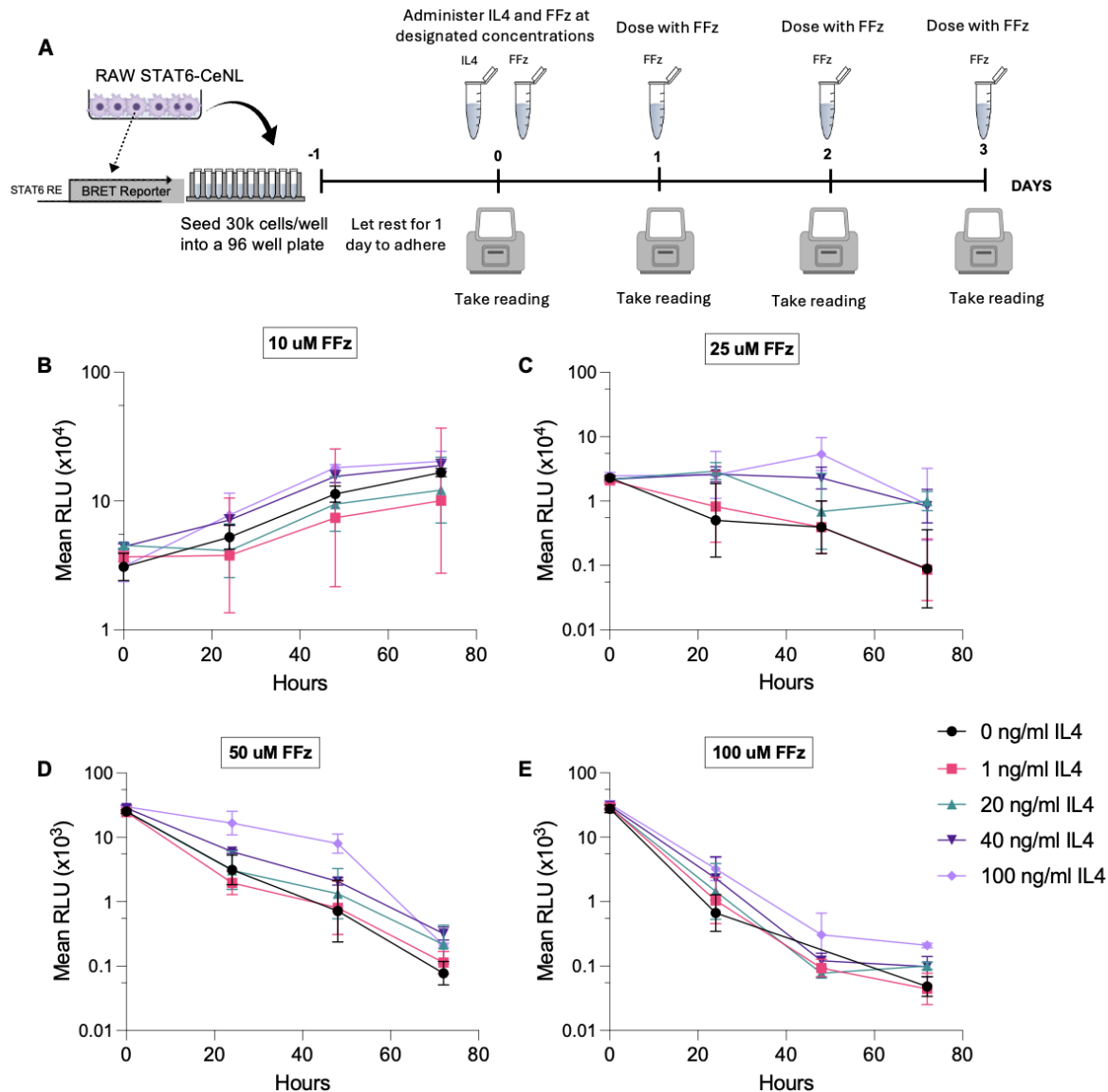


Figure 4 Optimization of Fluorofurimazine Substrate with Varying IL4 Concentrations.

A) Experimental Setup 30,000 RAW 264.7 STAT6-CeNL macrophages were seeded in a 96-well plate and allowed to rest for 24 hours before adding varying concentrations of IL4 to induce STAT6 activation. Cells were incubated for 72 hours and measured daily starting at Day 0 utilizing varying concentrations of Fluorofurimazine to catalyze the fluorescence reaction that was measured with the Luminescence 96 Byonoy (BiotekPlate reader) for B) The Effect of IL4 concentration on Mean RLU with a FFz concentration of 10 μ M added before each measurement C) The Effect of IL4 concentration on Mean RLU with a FFz concentration of 25 μ M added before each measurement D) The Effect of IL4 concentration on Mean RLU with a FFz concentration of 50 μ M added before each measurement E) The Effect of IL4 concentration on Mean RLU with a FFz concentration of 100 μ M added before each measurement. Sample size. N=4 per experimental group. Control = only RAW cells without IL4 polarization media added. Sample Size. N= 4 per experimental group. **Control= Only RAW 264.7 STAT6 Macrophages**

At the lower intermediate condition of 25 μ M FFz, gradual increases in bioluminescence intensity through 48 hours, followed by a sharp decrease, were observed when

activated with the highest IL4 concentration (100 ng/mL) (**Figure 4C**). However, these 25 μ M FFz-supplemented macrophages in 20 or 40 ng/mL IL4-activated conditions saw a decrease in bioluminescence intensity between 24 and 48 hours, with an immediate decline for 1 ng/mL IL4-activated conditions. Therefore, while bioluminescence signal intensity was observable for higher IL4 concentrations in 25 μ M conditions, signal duration was limited to a day. In addition, the Mean RLU was lower in 25 μ M FFz conditions than 10 μ M conditions, suggesting that immediate cell death was occurring even while signal intensity was increasing, as less cells were available to give off a readable signal.

FFz concentrations of 25, 50 and 100 μ M failed to read the full bioluminescent signal from IL4 dosage over a 3-day time course, potentially due to cell death from FFz toxicity over repeated substrate administration (**Figure 4D and 4E**). This conclusion is further confirmed through the observation of a 10-fold signal reduction in FFz concentrations in the 50 and 100 μ M FFz supplemented cells that was not observed in the moderate 25 μ M FFz administration regime. However, this concentration still induced cell signal decline after 24 or 48 hours, depending on IL4 condition, although signal intensity was not as significantly reduced compared to higher concentrations. In addition, STAT6 signaling initially increased as macrophages were polarized by IL4, but repeated FFz dosing reduced cell concentration and, therefore, signal output. Further, the 50 and 100 μ M FFz dosage regimes showed an immediate, marked decrease in signal intensity that was not observed for all IL4 stimulation regimes in the 25 μ M FFz condition. This decline in signal intensity shown by the slope of the graphs was sharpest for the highest FFz

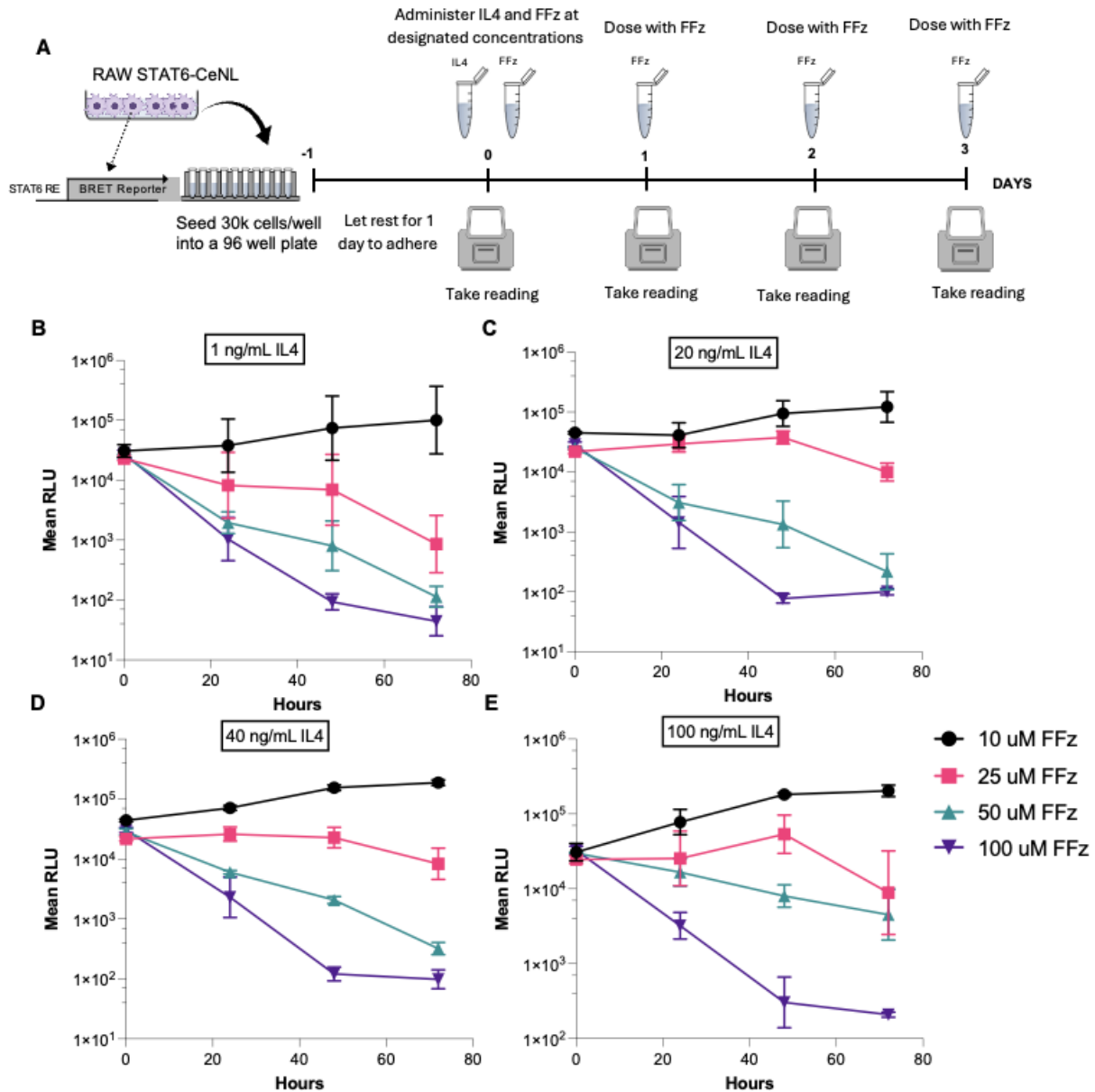
dosage regime, with all IL4 doses across all FFz conditions decreasing across a 3-day period.

Overall, 10 μ M FFz supplemented cells were able to measure signal intensity, highlighting increased concentration dependent IL4 mediated macrophage polarization monitored through STAT6 activation through 72 hours that was not seen in other, higher FFz administered conditions. This suggests that 10 μ M FFz is a sufficient substrate concentration to quantify the true scope of STAT6 activation.

Determination of Optimal Timing and Length of Dosing

Cell bioluminescent activity continues to increase for all IL4 concentrations through 72 hours, showing that macrophage polarization and cell activity is maintained through this point without additional IL4 or media supplementation (**Figure 5**). However, daily FFz dosages were necessary to catalyze the NanoLuc-FFz enzyme-substrate reaction to produce a bioluminescent signal at each 24-hour measurement point. Cells are therefore maintaining their polarized, STAT6 activated phenotypes over the course of 72 hours without continuous IL4 administration after the initial dosage.

Figure 5 Optimization of FFz Substrate Varying Fluorofurimazine Concentrations



A) Experimental Setup 30,000 RAW 264.7 STAT6-CeNL macrophages were seeded in a 96-well plate and allowed to rest for 24 hours before adding varying concentrations of IL4 to induce STAT6 activation. Cells were incubated for 72 hours and measured daily starting at Day 0 utilizing varying concentrations of Fluorofurimazine to catalyze the fluorescence reaction that was measured with the Luminescence 96 Byonoy (BiotekPlate reader) for B) The Effect of FFz concentration on Mean RLU with an initial IL4 concentration of 1 ng/mL IL4 added C) The Effect of FFz concentration on Mean RLU with an initial IL4 concentration of 20 ng/mL IL4 added D) The Effect of FFz concentration on Mean RLU with an initial IL4 concentration of 40 ng/mL IL4 added E) The Effect of FFz concentration on Mean RLU with an initial IL4 concentration of 100 ng/mL IL4 added. Sample size. N=4 per experimental group. **Control = only RAW cells.**

Keeping FFz constant, IL4 concentrations are graphed according to hours of incubation vs mean bioluminescence intensity to the fourth or third power (**Figure 5**). Across the 72-hour incubation period, only macrophages measured following 10 μ M FFz supplementation experienced increasing bioluminescent signal. This denotes continuous increasing cell polarization measured by STAT6 reporter activation, where higher concentrations of IL4 increase the signal output. This aligns with predictions of a concentration IL-4 dependent increase in signal intensity marking increased macrophage polarization (Pajarinen et al., 2015). Results were statistically significant compared to control conditions where STAT6 was not activated via IL4, showing a 1.4- to-2.6-fold increase in bioluminescence activity compared to controls at 24 hours. Signal intensity compared to controls only increased at the next two points showing a range of 3.2-to-5.8-fold bioluminescent increase at 48 hours and a 4.3 to 6.5-fold mean bioluminescent intensity at 72 hours. Across the two highest (50 and 100 μ M) FFz concentrations, signal markedly decreased from the initial signal reading through the end of the three days, suggesting potential cell cytotoxicity caused by high FFz dosages. At 25 μ M FFz, only the three highest concentrations of IL4 were able to induce minute signal increase. Mean RLU increased through 48 hours for the 20 and 40 ng/mL concentrations and by 72 hours for the 100 ng/mL IL4 concentration (**Figure 5B-E**). This could be attributed to higher IL4 dosages increasing macrophage polarization denoted by bioluminescence activity at a level high enough to balance the potential cell death occurring from FFz cytotoxicity.

Macrophage bioluminescent activity followed an almost IL4-dependent macrophage activation pattern, where higher IL4 dosages (100 and 40 ng/mL IL4) showed the

highest signal intensity across all FFz substrate concentrations. This was less clearly observed for lower doses with a smaller difference in concentration (1 and 20 ng/mL). Overall, these graphs suggest that the optimal FFz concentration is 10 μ M, with supplementation every 24 hours necessary to achieve full bioluminescent signal while minimizing cell death. IL4 dosages between 40 and 100 ng/mL showed consistent, increasing signal intensity throughout a 3-day time course. As other studies have shown success of 20 ng/mL IL4 dosages in induction of M2 phenotypic polarization, IL4 doses ranging between 10-40 ng/mL were utilized (Ishida et al., 2023; Mia et al., 2014). Therefore, for following experimental bioluminescent-based readouts, this concentration and dosage schematic was maintained.

Chapter 3 Fibroblast/ Macrophage Coculture 2D

Significance

Fibrosis is characterized by the accumulation of extracellular matrix (ECM) due to sustained inflammatory stimulation, leading to prolonged fibrotic stimulation and tissue dysfunction. Macrophages can exert both pro- inflammatory (M1) and anti-inflammatory (M2) characteristics, thereby playing a key role in inflammatory signaling (Merz et al., 2021; R. Su et al., 2025). During tissue repair, the polarization of macrophages into an M2 phenotype promote the secretion of pro-fibrotic factors, leading to the activation and proliferation of fibroblasts that increase collagen deposition (Behmoaras et al., 2025; Y. Jiang et al., 2024b). Therefore, targeting macrophage polarization may serve as a therapeutic target for fibrosis. The STAT6-mediated signaling pathway activated by IL4 can be used to measure M2a polarization (Zheng et al., 2024). As current phenotyping techniques rely on spatial transcriptomics, dynamic real-time, temporal measurements of macrophage polarization are lacking, leading to an insufficient picture of the involvement of macrophages in fibrosis. The use of Bioluminescence Temporal Spectrometry (BTS) to measure the bulk luminescence intensity of a STAT6-reporter element in macrophages can be used to quantitatively measure the activation of STAT6 (Zheng et al., 2024). Therefore, the use of a luciferase reporter system to monitor M2 polarization can be used to dynamically monitor macrophage heterogeneity in a space and time-dependent manner, leading to deeper understandings of fibrosis disease mechanisms.

Fibrosis and Macrophage Crosstalk

The fibrotic microenvironment involves a complex interaction between fibroblasts, macrophages, ECM components and secreted cytokines that exacerbates the inflammatory fibrotic environment (**Figure 6**). This underscores the complexities of macrophage/fibroblast interactions that are involved in diseases such as fibrosis. Therefore, an environment incorporating fibroblast and macrophage cross talk would allow for a more complete, accurate understanding of the progressive inflammation that characterizes fibrosis.

Repetitive or sustained inflammation or dysregulation in the wound-healing response can result in the progressive deposition of collagen surrounding or within damaged or inflamed tissue (T. A. Wynn & Ramalingam, 2012b). This persistent inflammation resulting in the deposition of ECM is termed fibrosis, and can result in scarring, organ dysregulation, and death in many diseases while serving as a pathological feature in chronic immune conditions. Fibroblasts, cells found in ECM associated tissues and organs, are activated during wound repair to synthesize and secrete ECM molecule, exhibiting phenotypically diverse phenotypes in their normal and active state following tissue damage (McAnulty, 2007; Younesi et al., 2024). Myofibroblasts, one such activated fibroblast phenotype, are central to the diverse fibrotic forms, key to collagen and fibrotic tissue remodeling (Gabbiani, 2003; T. A. Wynn & Ramalingam, 2012b). Macrophages are also crucial, with the recruitment of monocytes and differentiation into macrophages in damaged or dysregulated tissue contexts suggested to promote damage and fibrosis (Park et al., 2022a). This highlights the importance of considering environmental context in the monitoring of disease progression, specifically in fibrosis.

Further, macrophage infiltration into inflamed tissue has been correlated to the strength of damage and fibrosis (Braga et al., 2015; Eardley et al., 2006). Therefore, considering how macrophage presence affects fibrotic environments is critical to elucidating the intricate cellular dynamics of fibrosis.

Using Monocyte STAT6 biosensors to measure features of Fibrosis

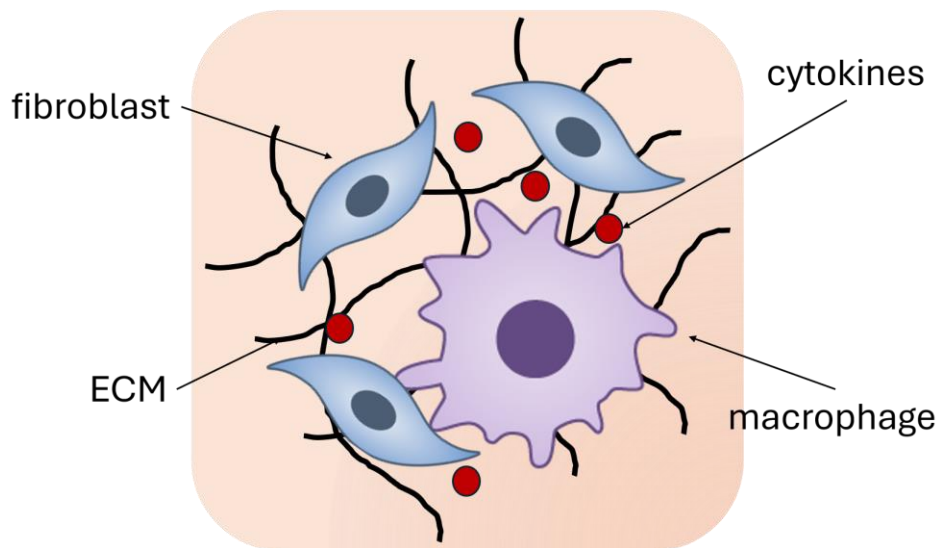


Figure 6 Cellular and Stromal Components of the Fibrosis Microenvironment

The fibrosis microenvironment is a complex network of fibroblasts, macrophage, ECM and cytokine interactions that acts as a self-sustaining environmental niche driving organ dysfunction.

As with other diseases, inflammation can contribute to the release of pro-fibrotic cytokines, chemokines and growth factors by inflammatory and activated resident cells that contribute to the state of fibrotic disease. Such stimuli affect the regulation of fibroblasts and the production of ECM components (Wick et al., 2010). Specifically, T-helper-2 cytokines (Th2) IL4 and IL-13 are both involved in activating the IL-4/STAT6 signaling pathway, driving the differentiation and recruitment of fibroblasts to tissues along with driving the polarization of macrophages into its M2 phenotype (Borthwick et al., 2013). Therefore, these cytokines are seen as effector cytokines in fibrosis, inducing

collagen synthesis of fibroblasts which is largely implicated in fibrosis. Activated macrophages are involved in this signaling paradigm due to their regulation of inflammatory ECM processing, shown in the resolution of liver fibrosis (Fallowfield et al., 2007; Wick et al., 2010). The release of inflammatory signals by macrophages has also been found to slow tissue and wound repair, leading to increased fibrosis, suggesting that macrophage specific signaling is key in progressive inflammation associated with fibrosis (Mori et al., 2008). M2 macrophages, activated via IL-4, have been implicated in pro-fibrotic cytokine production, suppression of the M1 response and recruitment of fibroblast cells (Braga et al., 2015; Zhu et al., 2017). M2 macrophages are associated with the peak pro-fibrotic immune response, secreting large amounts of pro-fibrotic factors such as TGF β , PDGF, VEGF, IGF-1 and Galactin-3 that promote myofibroblast proliferation and activation as well as the buildup of ECM components (Lech & Anders, 2013; Pilling et al., 2009). Therefore, M2 macrophage-myofibroblast cross talk can exacerbate inflammation, leading to fibrosis. However, M2 sustained activation has also been suggested to ameliorate fibrotic ECM synthesis, painting a complex picture of M2 function in fibrosis (Murray & Wynn, 2011a; T. A. Wynn & Ramalingam, 2012b). Therefore, elucidating the role of macrophages in an environment with fibroblasts to resolve time and spatial activation cues would inform current knowledge of the role of macrophage and fibroblast cross talk in fibrosis to allow temporally and spatially targeted treatment for fibrosis.

Fibrosis: Macrophage 2D Coculture Set Up

We predicted that the incubation of fibroblasts and macrophages would induce concentration-dependent activation of the M2 anti-inflammatory pathway, implicated in

fibrosis. This would be observable by the concentration-dependent activation of STAT6, a transcriptional activator associated with M2 polarization.

To simulate an environment of macrophage: fibroblast crosstalk, RAW 264.7 STAT6 macrophages were seeded at 10k cells/ well in 100 μ L of DMEM, constant across the 96 wells. This was necessary to normalize signal output to constant macrophage cell number input, whose bioluminescence activation via the STAT6 reporter was being measured. Following a 24-hour rest period to ensure adherence of RAW 264.7 CeNL-STAT6 macrophages, 3T3 fibroblasts were added at varying concentrations to obtain 0.5:1, 1:1, 2:1, 4:1 and 8:1 fibroblast to macrophage ratios. Activated fibroblast media obtained from the spun-down cells was added to the final column to determine the effect of cell-cultured media on macrophage STAT6 activation (**Figure 7**). Changing the ratios of macrophage: fibroblasts was necessary to model the dynamic shifts in immune cell composition across phases of wound healing and fibrotic conditions (Enriquez-Ochoa et al., 2026). Further, the accumulation of myofibroblasts as well as the number and type of macrophages are seen as crucial for the pathological fibrotic process (X. Li et al., 2024). Therefore, understanding the effect of varying ratios of these two main effector cells is critical towards understanding the process by which fibrosis occurs.

The control condition was not supplemented with fibroblast cells or activated media; instead, it contained 200 μ L of DMEM culture media. All conditions had 16 replicates except for the 8:1 ratio and activated fibroblast media conditions, which had n=8. On day 0 and each subsequent day, 10 μ M FFz, which was found to be the optimal concentration for long-term bioluminescence readings, was added to stimulate cell

reporter activation and obtain bioluminescent readings. At day 3, 100 μ M of DMEM media was removed and replaced with fresh media.

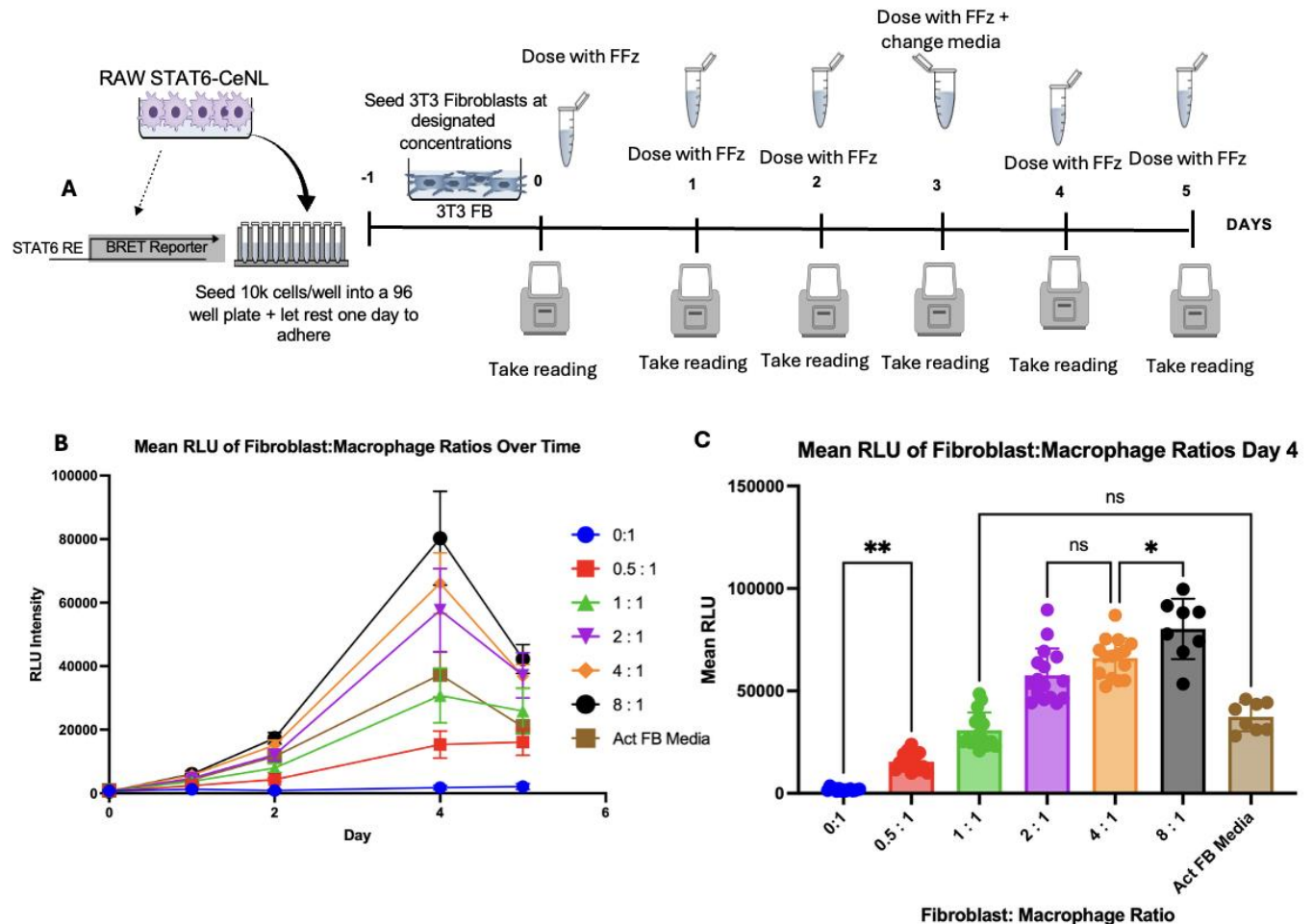


Figure 7 2D Co-culture of RAW STAT6 CeNL Macrophages with Varying Concentrations of 3T3 Fibroblasts (FB) to Assess the Effect of FB on M2 polarization

A) 2D Co-culture of RAW 264.7 STAT6 CeNL Macrophages with Varying Concentrations of 3T3 Fibroblasts (FB) to Assess the Effect of FB on M2 polarization. Experimental Setup: 10,000 RAW 264.7 STAT6-CeNL macrophages were seeded in a 96-well plate and allowed to rest for 24 hours before adding varying concentrations of 3T3 FBs (or FB activated media) to assess their effect on M2 polarization. Cells were incubated for 5 days and measured daily utilizing 10 μ M Fluorofurimazine as optimized in previous experiments to catalyze the bioluminescence reaction to activate STAT6. Mean RLU signal output as a proxy for anti-inflammatory activation was measured with the Luminescence 96 Byonoy (BiotekPlate reader) daily after FFz substrate addition. B) Mean RLU activation over 5 days comparing experimental group. C) Column analysis for Day 4 of the time course experiment comparing experimental groups. Sample size. N=16 per experimental group excluding 8:1 and Act FB Media that had N=8. **Control = only RAW cells.** Statistical Significance: Two-Way Anova and TukeyPost-hoc test for 6B and One-Way Anova and TukeyPost-hoc test for 6C. *p<0.033, **p<0.002, ***p<0.001, ns (non-significant)

Results and Discussion

The bioluminescence intensity corresponding to each macrophage: fibroblast ratio is plotted as Relative Light Unit (RLU) intensity or Mean RLU (**Figure 7**). STAT6 bioluminescence gene expression was measured every 24 hours over a 5-day period after supplementation with 10 μ M FFz. Statistical analysis was performed with a 2-way ANOVA multiple comparisons test for **Figure 7B** and a one-way ANOVA multiple comparisons test for **Figure 7C**. The curve shows an increase in STAT6 bioluminescence intensity for all conditions through day 4 after which signal intensity fell. This could signify that at day 4, fibroblasts are no longer to induce macrophage anti-inflammatory polarization. However, these results could be due to cell death or cytotoxicity from long-term culturing or overgrowth. Alternatively, removal of fibroblasts from media following a media change at day 3 could have resulted in removal of polarization stimuli needed for STAT6 gene expression.

Between non-control experimental groups, significant differences in bioluminescence activity could be seen at 48 hours. This suggests that while fibroblast-mediated M2 activation occurs on a shorter, 24-hour time scale, density-dependent activation, signified by culturing macrophages with varying numbers of fibroblasts, occurs on a slower 24–48-hour time scale. Overall, based on the Tukey post hoc comparisons from the two-way ANOVA, the fibroblast: macrophage ratio had a strongly time-dependent effect on RLU output. Across days, the magnitude and significance of differences between ratios of cultured fibroblast: macrophages increased substantially, suggesting progressive fibroblast-mediated enhancement of the measured response over time. Looking specifically at day 4, all conditions were largely statistically significant between

each other ($p < 0.001$) excluding the 1:1 vs activated fibroblast media condition and 2:1 vs 4:1 which were both nonsignificant (ns), the 4:1 and 8:1 which had a p value of < 0.033 and the control and 0.5:1 ratio which had a p value of $p < 0.002$ (**Figure 7C**). This suggests that both at the upper and lower end of fibroblast: macrophage ratios that differences became unclear due to lack of variation in activation conditions with fibroblasts above a 4:1 ratio producing similar results to double concentration. Notably, the activated cell media was able to induce STAT6 activation to a level similar to the 1:1 fibroblast: macrophage ratio, suggesting that there is a media specific component rather than just cell specific inducing STAT6 activity. Therefore, both fibroblasts and its media contain components that trigger STAT6 activation, with low and high ratios making the differences in STAT6 activation less clear.

Result of Macrophage: Fibroblast Co Culture on STAT6 Activation

Analysis of fibroblast: macrophage 2D coculture conditions was carried out utilizing two-way ANOVA time-course analysis and one-way ANOVA/Tukey comparisons for specifically day four. The fibroblast–macrophage coculture demonstrates a strong, time-dependent enhancement of macrophage bioluminescent STAT6 reporter activity with a dose-dependent STAT6 activation up to a maximal ratio of fibroblast: macrophages (**Figure 7**). The day 4 analysis was chosen as a point of maximal activation across all culture conditions and is representative of a later-stage coculture environment where fibroblast-mediated signaling has had time to establish.

Overall, fibroblast presence significantly increased macrophage reporter activity in a ratio- and time-dependent manner, indicating that fibroblast-derived signals

progressively alter macrophage transcriptional activity during coculture (**Figure 7B**). At day 0, all groups displayed relatively low baseline bioluminescence, suggesting minimal immediate effects from coculture initiation. However, beginning on day 1, fibroblast-containing conditions demonstrated increased STAT6 reporter activity compared with macrophage-only controls, indicating that fibroblast-mediated macrophage modulation occurs rapidly following coculture establishment.

At day 1, all fibroblast-containing conditions showed significantly increased bioluminescence compared with the macrophage-only control (0:1), including 0.5:1, 1:1, 2:1, 4:1, and 8:1 fibroblast: macrophage ratios (all $p < 0.0001$). Activated fibroblast media also significantly increased macrophage reporter activity compared with the control condition ($p = 0.0002$), demonstrating that fibroblast-secreted soluble factors contribute to macrophage activation. The progressive increase in signal with increasing fibroblast abundance suggests a dose-dependent relationship between fibroblast presence and macrophage STAT6-associated transcription.

By day 2 and day 3, the magnitude of fibroblast-mediated enhancement increased further, suggesting that fibroblast–macrophage communication is cumulative rather than an acute response. Higher fibroblast ratios produced progressively greater reporter activity, with fibroblast-rich conditions (4:1 and 8:1) producing the strongest responses. However, STAT6 activation differences between these conditions were not observed at any point in this experiment. Therefore, a maximal point of STAT6 activation might be reached at the 4:1 condition, where fibroblast density higher than that provide no significant differences compared to fibroblast: macrophage ratios to a certain threshold.

However, more studies comparing different cell counts at both higher and intermediate dosages could make this finding more conclusive.

A time-dependent increase overall in STAT6 activation indicates that fibroblasts may actively remodel the macrophage signaling environment through sustained paracrine signaling, extracellular matrix interactions, or direct cell-contact-dependent communication. However, as activated fibroblast media was able to increase STAT6 activity, both cell-cell interactions and cell-media interactions are shown to produce measurable STAT6 activation. Therefore, while fibroblast cell number is implicated in STAT6 activation, so is fibroblast secretion, as media alone was able to activate macrophages along the STAT6 pathway.

By day 4, RLU values reached their highest overall levels, where all fibroblast-containing conditions and the activated media condition produced significantly higher reporter activity than the control condition (**Figure 7C**). Although most pairwise comparisons remained significant, differences among the highest fibroblast-containing groups began to plateau. The 2:1 and 4:1 conditions were no longer significantly different ($p = 0.4039$), nor were the 4:1 and 8:1 conditions ($p = 0.2652$). Only the comparison between the 2:1 and 8:1 groups remained significant ($p > 0.001$). The activated fibroblast media remained comparable to the 1:1 coculture ratios ($p = 0.4546$) (**Figure 7C**). These findings indicate that increasing fibroblast abundance beyond a certain threshold produced smaller variation in RLU output, as at higher ratios, the differences between their bioluminescence output remained similar. Alternatively, long term culturing dampens the effect of higher levels of fibroblast numbers cultured with

macrophages where well size and potential lack of media renewal providing suboptimal conditions for continued fibroblast: macrophage interactions.

A key finding in day 4 pairwise comparisons was that activated fibroblast media alone significantly increased macrophage reporter activity compared with macrophage-only controls, suggesting that soluble fibroblast-derived factors are sufficient to influence macrophage signaling. This can be seen in the ns difference between the activated fibroblast and 1:1 fibroblast: macrophage ratio. However, direct fibroblast coculture at ratios higher than 1:1 produced higher activation results in higher STAT6 activation. The significant differences between the 4:1 and 8:1 fibroblast coculture and activated media ($p < 0.001$) suggest that soluble factors alone do not fully account for macrophage modulation. Therefore, separate interactions are occurring between direct cell-cell contact and media and macrophage contact.

Looking at day 5, macrophage monoculture remained significantly lower than all fibroblast-containing conditions (all $p < 0.0001$), while differences among high-fibroblast ratios largely disappeared to an even greater extent than on day 4 (Figure 7C).

Comparisons between the 2:1 and 4:1 ratios ($p > 0.9999$), 2:1 and 8:1 ratios ($p = 0.3568$), and 4:1 and 8:1 ratios ($p = 0.1274$) were all nonsignificant, indicating that maximal activation had been achieved by approximately the 2:1 fibroblast: macrophage ratio. However, even differences amongst lower ratios, although still significant, were dampened, with 0.5:1 vs activated FB media, 1:1 vs 2:1 and 1:1 vs 4:1 showing slightly larger p values (0.0260, 0.0020, 0.003, respectively). This shows the declining M2

polarization activation from coculture with fibroblasts past day 5 without additional FB supplementation.

Across all days, the data demonstrate a fibroblast dose-dependent enhancement of the measured response over time. Control conditions containing only macrophages consistently produced the lowest RLU values, while increasing fibroblast proportions progressively increased activity. Early time points showed limited separation among coculture ratios, whereas by day 2, a stronger discrimination between experimental conditions was seen. However, no difference in a 4:1 or 8:1 ratio was observed throughout the course of the experiment. Collectively, these findings indicate that fibroblasts are a major driver of the measured response, suggesting that fibroblast activation scales with dose up to a certain concentration and that activated media can induce STAT6 activation and therefore induce M2 polarization. Together, this implicates a fibroblast mediated polarization paradigm for macrophages that can be used to resolve temporal signaling cues crucial in fibrosis.

Section 3 Co-Culture Conclusions

The progressive increase in STAT6-associated bioluminescence during fibroblast coculture suggests that fibroblasts actively promote a macrophage transcriptional state associated with alternative activation. Since STAT6 is a major transcription factor involved in M2-like macrophage polarization, these results suggest that fibroblast-derived cues activate anti-inflammatory M2 macrophage phenotype at a dose-dependent level. Importantly, the temporal pattern differs from an acute activation response. Rather than a rapid transient increase, macrophage STAT6 activity increased

progressively over several days, indicating that fibroblast-mediated effects likely require establishment of a remodeled cellular microenvironment. This supports a model in which fibroblasts influence macrophage state through sustained communication rather than a single activating stimulus.

Direct fibroblast–macrophage interactions likely enhance STAT6 through mechanisms such as membrane-bound ligand interactions, extracellular matrix signaling, cell-contact-mediated feedback. This agrees with literature where M2 macrophages exposed to fibroblasts enhance ECM deposition through upregulation of IL-4, IL-10 and IL-13 and activate fibroblast proliferation and collagen synthesis through producing TGF β , CTGF and CCL18. Less explored is the effect of fibroblast media alone, without cells present, in activating macrophages (Froom et al., 2025; Laskin et al., 2019; Lis-López et al., 2021). I show here that media alone can activate macrophages, suggesting a media-specific component of fibroblasts that drive macrophage STAT6 activation and M2 polarization. Overall, these findings demonstrate that fibroblasts significantly enhance macrophage STAT6-dependent signaling through both soluble and contact-dependent mechanisms, supporting the role of fibroblast–macrophage interactions in regulating macrophage polarization and tissue microenvironment remodeling.

Chapter 4: Organoid co culture model with STAT6

Abstract

The tissue microenvironment involves a complex interplay of diverse cells, structures and signals that is difficult to capture with a 2D in vitro environment. Further, cell cultures are a crucial tool in improving knowledge of diverse biological processes in homeostasis and disease. Therefore, performing 3D co culture experiments are crucial towards capturing the true, complex, and dynamic cellular environment to provide more relevant and informed analysis of cell-cell and cell-environmental interactions for higher in vivo application. Further, optimization of 3D cell culturing parameters, macrophage seeding density and bioluminescent plate reading analysis are crucial towards analyzing 3D culture readouts and reducing outside effects on STAT6 activation. Therefore, macrophage reporter cell seeding density, plate reader and culturing method optimization was performed to ensure reliable, STAT6 activation as a proxy for macrophage polarization in response to experimental conditions.

Benefits of a 3D Cell Culture Model to Track Macrophage Phenotypic Changes

The tissue microenvironment is made up of a complex, dynamic three-dimensional environment of a variety of cells, blood vessels, signals and chemicals that dictate cellular fate and driving cell homeostasis or disease progression. Recapitulating this dynamic cellular microenvironment in vitro is important to inform true biological in vivo

processes. While monolayer co culture methods help to recreate dynamic cell-cell dynamics and signaling paradigms, the flat, limited tissue architecture limits physiological translation (Cacciamali et al., 2022). 2D co cultures fail to mimic in vivo cell-cell and extracellular interactions, crucial in performing experiments to resolve both space and time dependent cellular cues in diseases such as fibrosis that largely occur through cell-cell contact signaling regimes (Kapałczyńska et al., 2016). Further, changes in cellular morphology due to 2D cell culture reduce their phenotypic diversity, influencing function, structural organization, cell secretion and signaling (Nelson & Bissell, 2006; Petersen et al., 1992). Therefore, 3D cell culture systems have been developed to better mimic physiological structures, properties and conditions to provide more representative findings and responses. Crucially, 3D cell cultures are able to recapitulate the structure of the Extracellular Matrix (ECM) composed of a scaffold of fibrillary and accessory proteins as well as adhesive structural and biochemical support molecules (Habanjar et al., 2021a). As fibrotic inflammation is driven by excessive ECM deposition and its interaction with both macrophages and fibroblasts, the ability for the 3D cell culture system to recapitulate this structure is crucial in elucidating the true macrophage and fibroblast temporal and spatial cross talk in fibrotic development.

3D cell culture models are either scaffold-based with natural or artificial scaffolding material or scaffold free, such as spheroids (Kapałczyńska et al., 2016). Spheroids are self-assembling cell aggregates of one or more cell types that have been found to recapitulate the 3D environment in diseased conditions due to their recapitulation of cellular heterogeneity, cell signaling pathways, cell-cell/cell-ECM pathways, gene expression patterns and morphology (Rodrigues et al., 2021). Spheroids can be formed

through seeding onto nonadhesive- hydrogels without ECM influence to encourage cell-cell binding and self-assembly. These micromolds, which are commercially available, produce high numbers of homogenous cell spheroids which recapitulate the structural and functional roles of in vivo tissue for biological applications (Białkowska et al., 2020; Habanjar et al., 2021b).

Macrophage and fibroblast spheroid formation in a 3D co-culture model would increase physiological relevance by more accurately reproducing the fibrotic microenvironment. Therefore, bioluminescence outputs from STAT6 activation upon macrophage and fibroblast incubation would better inform therapeutic interventions and better translate to in vivo fibrotic progression. Crucially, fibroblast only and macrophage-fibroblast spheroids were successfully fabricated in non-adhesive hydrogel molds using Mold Master from Microtissues (Tan et al., 2020a). However, the formation of spheroids was not accompanied by real time, temporal tracking of STAT6 macrophage activation, signifying macrophage polarization, along spheroid formation, limiting insights along the pro-fibrotic inflammatory progression that could inform diagnostic platforms. Therefore, having established a protocol for successful fibroblast and fibroblast: macrophage spheroid formation, I sought to apply BTS with STAT6-RE RAW 264.7 macrophages to track macrophage polarization in response to spheroid formation with and without fibroblasts.

Seeding Optimization of 3D Agarose Molds

A 3D scaffold-free, high throughout platform of spheroid formation was demonstrated by Tan et al utilizing an agarose-based 3D Petri Dish. A spheroid model provides an

environment for cell-cell interaction and communication that is crucially involved in fibrosis between fibroblasts and macrophages. This 3D platform for modelling human fibrosis was shown to reliably produce macrophage: fibroblast co cultures, but the use of end point measurements to analyze spheroid formation, macrophage polarization and fibroblast activation omits spatial and temporal data that could inform diagnostic platforms for fibrosis progression. Therefore, I sought to introduce our STAT6 macrophage cell reporter system into this agarose-mold based spheroid system in order to perform temporal and spatial resolved STAT6 activation signaling using BTS.

Two experiments, one for optimizing macrophage imaging and initial seeding density for monoculture spheroid formation and another for live cell imaging of activated macrophages in 3D spheroids were performed as outlined in **Figure 8** and **Figure 10**.

Briefly, non-adhesive agarose hydrogel molds were prepared using the Mold Master system (Microtissues, Inc., Providence, RI, USA) to create molds containing 96 circular recesses with hemispherical bottoms (800 μm diameter $\times$ 800 μm depth), which promoted consistent, small ($\sim 200 \mu\text{m}$) spheroid formation. For each mold, 330 μL of 2% autoclaved agarose was added to the Mold Master and allowed to gel at room temperature before carefully removing the solidified mold and transferring it into a well of either a 6 or 24 -well culture plate. Subsequently, 75 μL of RAW 264.7 STAT6 macrophages in DMEM media were added to each agarose mold. After allowing the cells to settle into the individual recesses for 15 minutes, an additional 2.5 or 1 mL of DMEM high glucose cell culture medium was added. Media was refreshed every two days throughout the duration of the experiment and cells were kept in a cell culture incubator at 37°C and 5% CO₂.

Over a 9-day time course experiment the Revolve Echo was used to monitor the formation of homogenous mononuclear RAW 264.7 STAT6 cell cultures in 3D Petri Dish Mold Masters by Microtissue (**Figure 8**). Six different culture conditions, with increasing macrophage cell numbers were utilized, ranging from 1400 to 324,000 cells per mold, as recommended by the manufacturing website.

Experimental Protocol for Seeding Optimization of 3D Agarose Molds

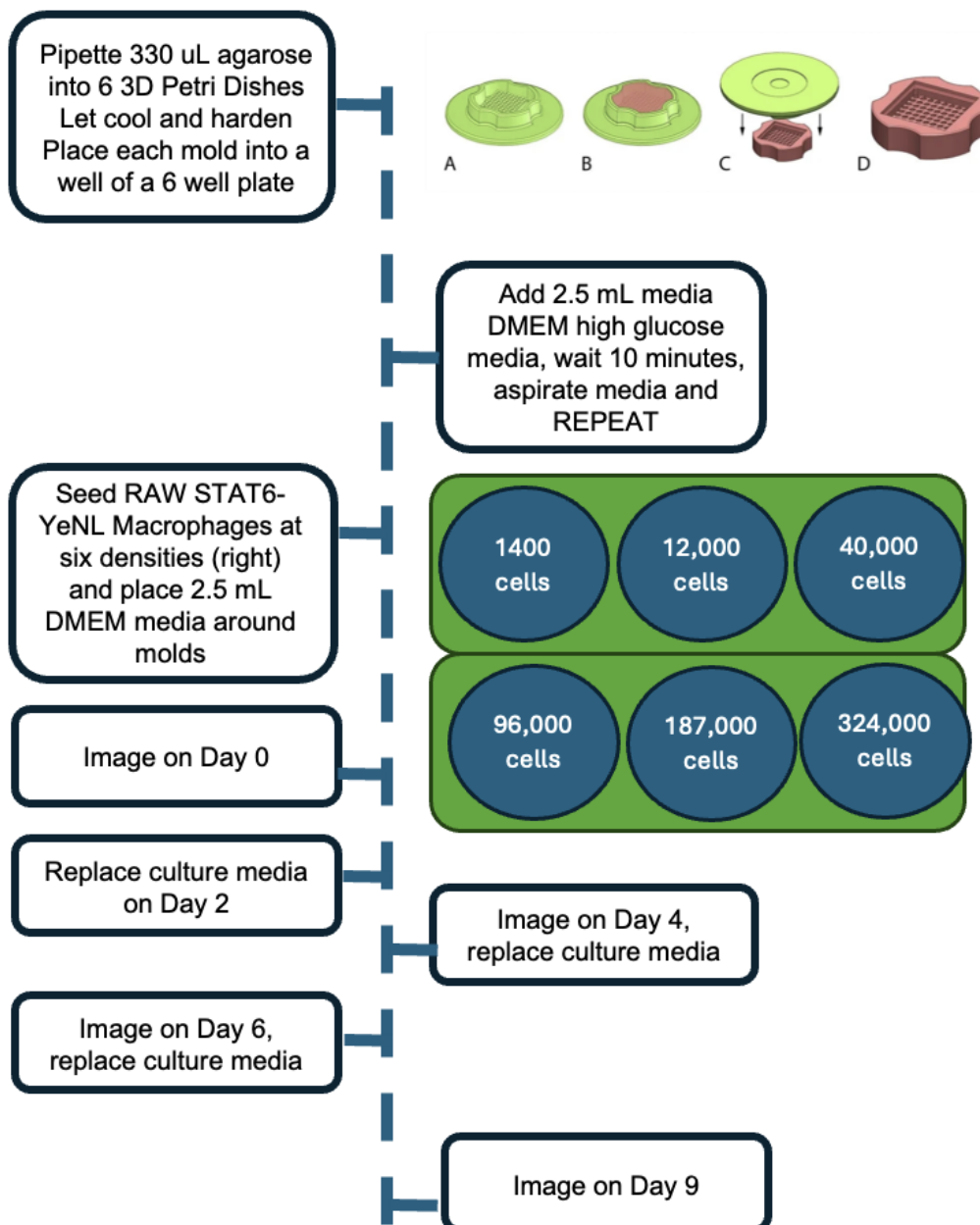


Figure 8 Spheroid Formation Utilizing Micromolds to Optimize Macrophage Seeding and Imaging Conditions

Steps were followed as outlined above and described in accordance with Microtissues, The Scaffold-Free 3D Cell Culture Platform for making uniform spheroids. The Silicone micro-mold contains 96 circular recesses to form small ~200µM spheroids.

Images were taken on the Revolve Echo Microscope at day 0, day 4, day 6 and day 9 (**Figure 9**). At day 0, macrophages were seeded into 3D Petri Dish Micromolds and imaged. Cells were seen in each seeding condition and localized to recesses for each seeding density as shown by day 0 images. By day 4, spheroids were seen in 12,000 cells and up conditions, noted by the clustering of cells in recesses of the molds. Notably, density dependent increases in spheroid sizes were seen particularly between 1400, 12000 and 40,000 cells per mold. 96,000- 324,000 cells per mold conditions showed size differences from the previous seeding densities, but less clear differences between each other. By day 6, 1400 cells per mold conditions showed sparse, nonuniform spheroid formation while 12,000 cells per mold spheroids failed to grow larger than their size on day 4. The seeding conditions for 40,000 and above showed larger spheroids that filled the diameter of the recesses, with 40,000 cells per mold showing smaller spheroids than the three higher seeding densities. However, cells grew beyond mold recesses, spreading throughout the mold surface and adhering onto the well bottom plate by day 6 for the 187,000 and 324,000 seeding densities. While spheroids were predominantly darker, highlighting 3D architecture, the cells cover the surrounding area of the recesses, suggesting that cell proliferation overrode the ability of the molds to contain cells to mold recesses to capitalize on cell aggregation and 3D spheroid formation. By day 9, 96,000 and above seeding densities were unresolvable on the microscope, as macrophages were overgrown and spreading beyond the molds, no longer forming spheroid shapes. 1400 and 12,000 cell/ mold conditions featured a few, non-uniform 3D spheroids while 40,000 cells/mold still contained 3D spheroids, but cell overgrowth beyond the mold recesses were seen.

Overall, formation for 12,000 and above seeding conditions were seen by day 4. This aligns with other studies that demonstrate the formation of mature spheroids utilizing the microtissue mold by day 5 (Tan et al., 2020b). By day 4, concentration dependent increases in spheroid sizes were seen from 1400 to 187,000 seeding conditions, with little spheroid sizes differences between the 187,000 and 324,000 seeding conditions. Between day 4 and 6, 40,000-324,000 seeding conditions showed increases in spheroid sizes, with the 187,000 and 324,000 seeding densities showing cell spreading beyond mold recesses onto the other mold surfaces and onto the bottom of the plate. By day 9, while 1400 and 12,000 cells/well conditions showed resolved spheroids, the spheroids were nonuniform across the mold. The 40,000 cells/ well condition showed cell overgrowth, with the three highest seeding conditions showing large amounts of cell overgrowth that made the detection of spheroids amongst overwhelming cell proliferation difficult to resolve. While spheroid formation was seen across each condition by day 9, spheroids were non-uniform across all cell seeding densities in the mold. Timing and size dependent spheroid formation varied across seeding conditions. However, the 40,000-187,000 seeing conditions showed reliable increases in spheroid diameter across days, with the highest seeding condition featuring high levels of cell overgrowth by day 6. Therefore, the three middle seeding densities were utilized for future imaging experiments, with day 3-day 6 seen as the crucial window for detecting spheroid formation for imaging and bioluminescent experiments.

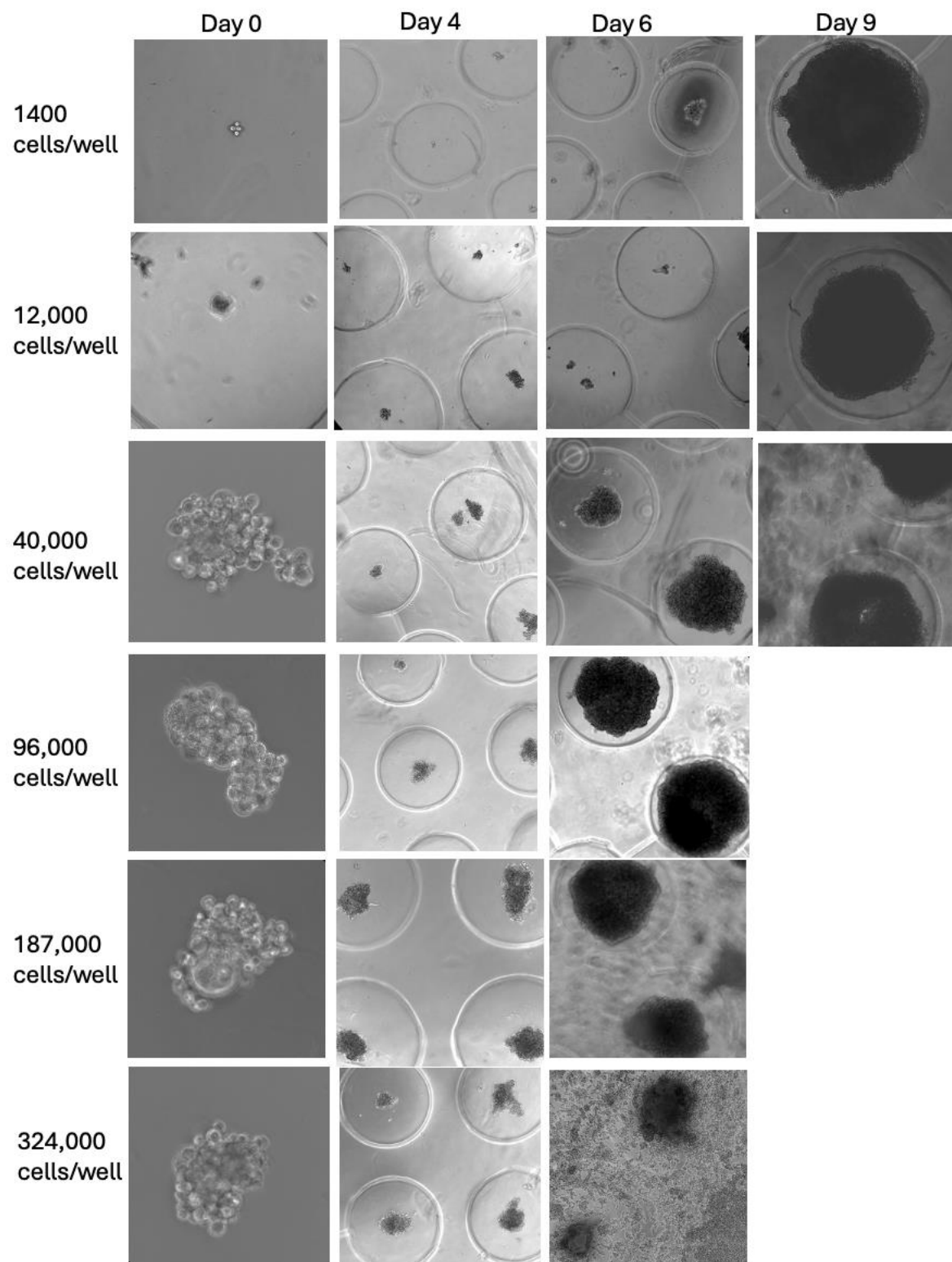


Figure 9 3D Petri Dish Agarose Gel Images for Macrophage Spheroid Optimization

RAW 264.7 STAT6 macrophages were seeded at 1400, 12,000, 40,000, 96,000, 187,000 and 324,000 cells per well in 3D Agarose Mold Master Petri Dishes provided by Microtissues as outlined previously. Cell images were on the Revolve Echo Microscope starting at Day 0 at 4x for 40,000-324,000 cells/ well and 20x for 12,000 and 14000 cells/ well to confirm macrophages were present and seeded in each mold. Images at were taken at Day 4, 6 and 9 following Day 0 images at 20x. The three highest seeding densities failed to produce clearly resolved images at Day 9 and are therefore removed from analysis.

3D Agarose Mold Fabrication for Live Imaging Analysis

A method to quantify and track the bioluminescence signal output via BTS was required to monitor macrophage STAT6 activation as a response to spheroid formation and fibroblast introduction. Due to the thickness of the agarose gel and the wide spatial distribution of macrophages across different recesses in one well, the Luminescence 96 Byonoy (BiotekPlate reader) was not an optimal tool for tracking bioluminescent cell signal. The LV200 microscope by Olympus provides long duration cell bioluminescent tracking with no background noise or autofluorescence an optimized light path to provide the highest resolution imaging (Ogoh et al., 2014). Its ability to spatially resolve bioluminescent signal within one spheroid could be employed to monitor STAT6 activation on a spheroid-by-spheroid level. Through the nine-day culture experiment with RAW 264.7 STAT6-YeNL macrophages seeded in 3D Petri Dish Agarose Molds, optimal seeding density to minimize cell overgrowth while maximizing stable, resolving spheroid formation was determined in addition to timing of spheroid development in monoculture conditions (**Figure 8**). Further, an initial experiment to develop consistent parameters for agarose mold preparation, well plate and media administration was necessary to ensure accurate and meaningful bioluminescent results.

Experimental Protocol for LV200 Imaging with 3D Agarose Molds

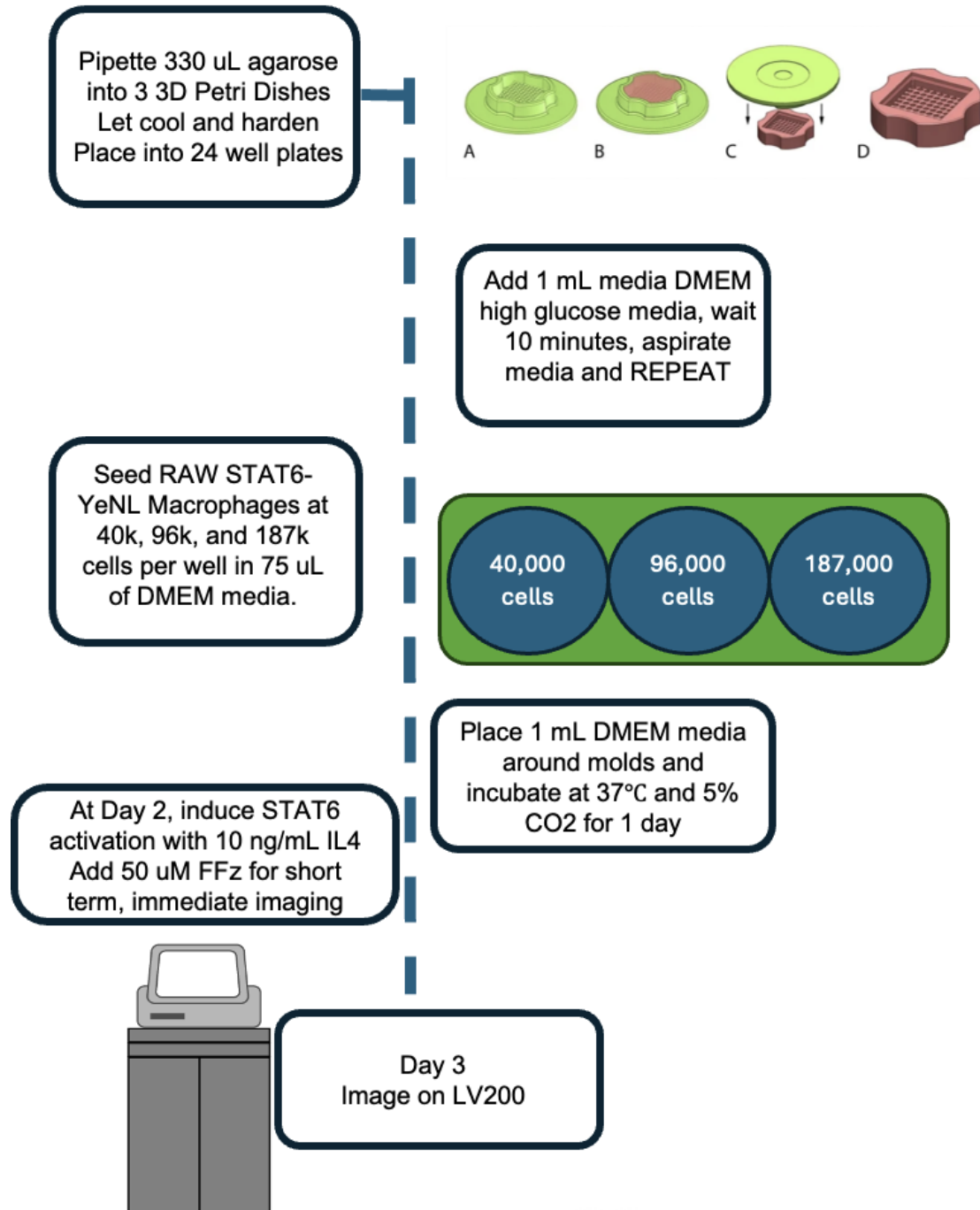


Figure 10 Macrophage Spheroid Fabrication for Live Image Analysis

Steps followed as outlined above in accordance with Microtissues, The Scaffold-Free 3D Cell Culture Platform for making uniform spheroids. The Silicone micro-mold contains 96 circular recesses to form small ~200 μ M spheroids. Spheroids were imaged after STAT6 activation via 10 ng/mL IL4 and 50 μ M FFz administration on the Olympus Live Cell Imaging System.

The experimental protocol for creating spheroids was the same as outlined in **Figure 8**, with 3 optimal seeding densities chosen by the previous experiment that showed minimal overgrowth over long term culturing as well as well-formed, consistent spheroids across the mold recesses. RAW 264.7 STAT6-YeNL macrophages were seeded at 3 densities (40,000, 96,000 and 187,000 cells) into molds that had been placed into 3 wells of a 24 well plate. Use of a 24 well plate rather than a 6 well plate would reduce the amount of DMEM media administered to each well while reducing the bottom surface area for cells to grow on if dislodged from molds. After 2 days incubation in 37°C and 5% CO₂ to allow for initial spheroid formation, STAT6 was activated through the administration of 10 ng/mL IL4. After 24 hours of activation via IL4, a high dose of FFz (50 μ M) was utilized to catalyze the bioluminescent reaction. Micromolds were placed in an imaging dish and imaged through the bottom of the mold on the Olympus Live Cell Imaging System (LV200) and inverted so that a layer of agarose did not disrupt the clarity of the optical path (**Figure 10**).

However, bioluminescence signal was unable to be detected by the LV200 following IL4 and FFz administration despite the confirmation of macrophage monoculture spheroid formation on the Revolve Echo Microscope. As this could be due to the thickness of the agarose mold, the mold was inverted on the glass chamber microscope plate to image without the layer of agarose that might have been disrupting signal intensity. Despite inverting the mold, a bioluminescent signal was still unable to be resolved. This could be due to a large distance between the mold bottom and the imaging lens, media

distortion, or disruption of spheroids due to flipping the mold on the plate. Overall, the failure to detect a bioluminescent signal limits the application of this spheroid culture mold master modality to resolving real time, temporal and spatial bioluminescent cues for the monitoring of STAT6 activation and inflammatory, pro-fibrotic progression. Therefore, another 3D culture modality was needed to be able to provide a quantifiable bioluminescent signal output over time while facilitating long term fibroblast: macrophage cell survival and crosstalk.

Utilizing Ultra Low Attachment U Bottom Plates for Bioluminescence

Temporal Spectroscopy

Despite successfully forming monoculture RAW 264.7 STAT6 macrophage spheroids, 3D Agarose Petri Dish Molds from Microtissues were unable to allow for detectable bioluminescent signal. This prevented real time, spatially and temporally resolved details on macrophage polarization which was a required component of our 3D cell culture system. Further, while concentration dependent increases in spheroid diameter were observed, spheroids were not uniform in size. Further, large cell overgrowth onto both the well plate bottom and spheroid mold beyond the recesses disrupted spheroid formation and observation. Therefore, a new 3D culture model to create reproducible, uniform spheroids was needed to provide detectable bioluminescent signal over long term culturing conditions.

96 Well Round U Bottom Plates have optical clear bottoms with black opaque microplate bodies to prevent well to well cross talk while enabling reading of bioluminescent signal output from each well. Importantly, the Ultra-Low Attachment

(ULA) surface allows for rapid, uniform and reproducible 3D spheroid formation for high throughput screening. Having been used to assess tumor spheroid formation and fibrosis, these plates are well suited to analyze co culture effects on spheroid formation (Ivanov et al., 2014; Song et al., 2021).

Effect of Macrophage Seeding Density on Bioluminescence Activity

While 96 Well Round U Bottom Plates have been previously validated to reliably form 3D spheroids, it is necessary to optimize seeding density and plate reading to ensure that the added dimension and different shape formation were not impacting bioluminescent output. (Hurrell et al., 2020; Xing et al., 2024). As excessive macrophage cell growth could be a cause of cell cytotoxicity or altered signal intensity, measuring the bioluminescence output of macrophages at different seeding densities over time would highlight the effect of macrophage cell growth on cell signal and viability. Plating densities have a large effect on macrophage morphology and cell profile. Changes in density was found to affect transitions of macrophages between pro-inflammatory and anti-inflammatory phenotypes, affecting their morphology, transcriptional profile, surface marker expression, and type and levels of cytokine and chemokine secretion (Vaughan-Jackson et al., 2022). In RAW264.7 cells, spatial confinement of macrophages due to high density plating caused suppressed inflammation, highlighting the potential for excessive macrophage growth to disrupt M2 polarization and therefore bioluminescence (Jain & Vogel, 2018). Further, macrophages derived from higher density cultures were found to produce less proinflammatory cytokines such as IL-6, IL-12 and TNF-alpha, were less phagocytic and had differential M1 and M2 gene expression upon IL4 activation (C. Lee & Hu, 2013). These studies

underscore the importance of monitoring macrophage signal intensity for different seeding densities across a longer time period to ensure the maintenance of signal viability and level.

To determine how macrophage cell growth effected signal levels via bioluminescence in long term culturing conditions, eight different macrophage seeding densities were plated and measured after polarization media was added across 4 days. STAT6 RAW CeNL Macrophages were plated at either 1k, 5k, 10k, 20k, 30k, 50k, 100k, or 150k cells per well at day -1 in 100 μ L of DMEM media supplemented with 10% FBS and 1% PenStrep. Cells were allowed to rest for 24 hours to adhere to plate before polarization media was added. 10 ng/mL of IL4 was added with 10 μ M FFz on day 0 with measurements immediately taken on the Luminescence 96 Byonoy plate reader. 10 μ M FFz was added every 24 hours before measurements were taken over 5 days. At day 3, 100 μ L of old culture media was changed for 100 μ L fresh media and measurements were taken. Crucially, measurements were taken from the top and bottom to determine the optimal plate reader position for 3D organoid culture models (**Figure 11**).

The purpose of this experiment was twofold: to optimize macrophage seeding density over long-term culturing conditions and to determine the maximal seeding density that would provide increasing, reliable signaling to pinpoint potential mechanisms of cytotoxicity in long-term culturing conditions due to macrophage cell overgrowth.

Further, comparisons between bottom-up and top-down readings with the Byonoy would illuminate the optimal well plate configuration for RLU measurement.

Interpretation of Bottom-Read Out 3D Macrophage Seeding Densities Through STAT6 Bioluminescence

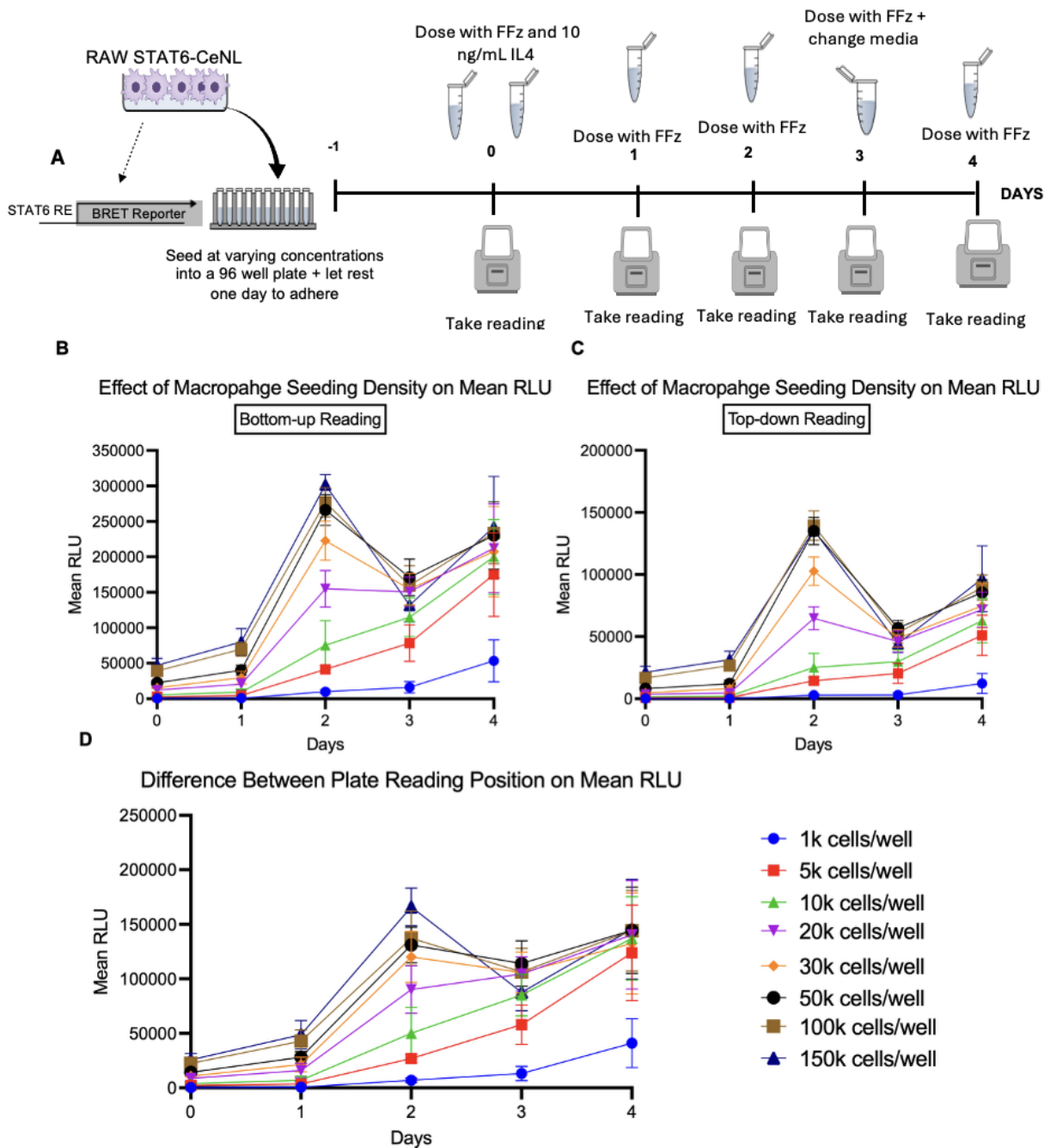


Figure 11 3D Macrophage Spheroid Optimization in U Bottom Plates

A) Experimental schematic of 3D culture of RAW 264.7 STAT6-CeNL Macrophages. Briefly, cells were plated at varying concentrations into a Corning 96 Well Ultra Low Binding, U-Shaped Bottom Microplate for uniform, reproducible multicellular spheroid formation. BTS measurements were performed using Luminescence 96 Byonoy (BiotekPlate reader) and taken daily over a 4-day period following administration of 10 μ M FFz before each measurement. STAT6 activation as a proxy for M2 polarization was initiated by 10 ng/mL IL4 administration on Day 0. Results looking at the effect of macrophage seeding density on bioluminescence measured by Mean Relative Luminescence Intensity (RLU) was performed utilizing B) A Bottom-up plate reader position and C) Top-down plate reader position. D) The difference between the bottom up and top-down plate reader position is graphed over time. Sample Size. N= 8 **Control = only RAW cells. Statistical Significance: Two-Way Anova and TukeyPost-hoc test. * $p < 0.033$, ** $p < 0.002$, *** $p < 0.001$, ns (non-significant)**

This experiment evaluated how macrophage seeding density influences IL-4-induced STAT6 signaling over a 5-day culture period (day 0–day 4) using a bottom-read bioluminescence assay in a 96-well plate (**Figure 11B**). Because the plate was read from the bottom, the measured signal likely reflects both biological STAT6 activation and optical effects associated with increasing cell density, including greater numbers of reporter-expressing cells and potential signal attenuation at very high densities. The optimal seeding density should maximize signal while maintaining sufficient dynamic range to distinguish biologically meaningful changes. Densities that produce indistinguishable outputs despite large differences in cell number indicate saturation and reduced assay sensitivity. This is especially important in 3D cell culture, where organoid formation results in cell stacking that could disrupt macrophage cell bioluminescence readability. Statistical analysis reveals three distinct phases: an initial density-dependent phase (days 0–1), a signal amplification phase (day 2), and a saturation/convergence phase (days 3–4).

At day 1, the assay demonstrated a strong density-dependent increase in STAT6 reporter activity. The 1k cells/well condition was significantly different from all densities greater than 10k cells/well ($p < 0.05$), indicating that lower seeding densities generate substantially weaker responses. This is expected due to a lower number of macrophages per well that are present to generate the STAT6 signal. However, several

neighboring densities were statistically indistinguishable, including 5k vs. 10k cells/well ($p = 0.309$), 10k vs. 20k cells/well ($p = 0.077$), and 20k vs. 30k cells/well ($p = 0.430$). Likewise, 100k and 150k cells/well did not differ significantly ($p = 0.848$). Expected for seeding densities that are within 5k/10k apart, the lack of significance between the largest seeding densities, 100k vs 150k, suggests that at the highest seeding densities, cell confluence overwhelmed signal resolution even at the earliest time point. This could also suggest lack of substrate availability due to high macrophage cell number.

Day 2 exhibited the greatest degree of statistical separation and therefore represents the point of maximal assay sensitivity. Most density comparisons were significant, including 10k vs. 20k cells/well ($p = 0.0031$), 20k vs. 30k cells/well ($p = 0.0033$), and 30k vs. 150k cells/well ($p = 0.0004$). The emergence of significant differences across intermediate densities indicates that the assay was operating within its largest dynamic range.

Importantly, saturation became apparent among the highest-density groups. The comparisons between 30k and 50k cells/well ($p = 0.0587$), 50k and 100k cells/well ($p = 0.9665$), and 100k and 150k cells/well ($p = 0.1829$) were not significant. Despite nearly doubling the number of cells from 50k to 100k, no measurable increase in reporter activity was detected. This suggests that STAT6-mediated bioluminescence was no longer proportional to cell number above approximately 50k cells/well. While this paradigm could be due to macrophage cell number being larger than available substrate to catalyze the luciferin/luciferase reaction, the signal output continuing to increase at subsequent densities suggests other factors exist. From a statistical perspective, this could indicate a transition from a density-dependent regime to a density-independent

regime at higher cell densities. Additional cells contribute little measurable information, implying that assay sensitivity begins to plateau beyond 50k cells/well.

By day 3, the significance between experimental groups markedly decreased. While cultures at and below 5k cells/well remained significantly lower than most other groups, nearly all comparisons among densities ≥ 20 k cells/well were nonsignificant. Specifically, comparisons among 20k, 30k, 50k, 100k, and 150k cells/well all failed to reach significance ($p > 0.05$). This loss of statistical separation is particularly noteworthy because the mean signals remained relatively high. The absence of significant differences despite substantial differences in cell number indicates that the biological response had reached a plateau. Consequently, increasing macrophage density beyond approximately 20–30k cells/well no longer produced a statistically distinguishable increase in STAT6 activity.

At the final day 4 time point, the saturation effect became even more pronounced where the only significant comparisons observed involved the 1k cells/well condition. All densities from 5k through 150k cells/well were statistically indistinguishable from one another. For example, comparisons between 5k and 150k cells/well ($p = 0.486$), 10k and 100k cells/well ($p = 0.844$), and 50k and 150k cells/well ($p > 0.9999$) were all nonsignificant, despite featuring large differences in cells/well (**Figure 11B**).

These findings indicate complete convergence of the STAT6 reporter response across moderate- and high-density cultures. This could be a result of over confluence of cells as they continued to grow over long term culture, resulting in cell density proving low correlation to reporter output. Further, this highlights the importance of cell density seeding considerations when plating macrophages not only for cell health and

resolution but for obtaining accurate bioluminescent data over long time periods. Tight control of cell density in accordance with cell type is necessary to maintain cellular specific phenotypes and properties (Neurohr & Amon, 2020). Specifically, increased cell density was found increase collagen formation and cartilage formation in biomaterial application, which is important in studies in fibrosis where collagen production and cell-cell interactions are crucial towards elucidating the role of specific cellular components towards producing its chronically inflamed phenotype (Talukdar et al., 2011). Therefore, optimizing cell seeding concentration is necessary in isolating the effects of specifically macrophage/fibroblast interactions on STAT6 activation in fibrosis.

The statistical evidence from the bottom plate reading on the Byonoy suggests that the optimal seeding density lies between 20,000 and 50,000 macrophages per well for long-term, 3D cell culture, providing real-time, temporally resolved bioluminescence monitoring. Lower signal intensities for macrophage cell densities below 10,000 cells/well indicated reduced assay robustness and lower reporter output. Further, day 2 showed significant separation between 10k, 20k, and 30k cells/well, demonstrating that the assay remained responsive to changes in cell number within this range. Across days, comparisons involving 50k, 100k, and 150k cells/well were frequently nonsignificant despite large increases in cell number, indicating saturation of the measured response. Overall, the 20k–50k range maintained high signal intensity while avoiding the density-independent behavior observed at 100k–150k cells/well.

The disappearance of significant differences among high-density groups indicates that measurements become increasingly statistically dependent on assay saturation rather than cell number. In other words, once cultures exceed approximately 50k cells/well,

reporter output no longer scales linearly with macrophage number, reducing the assay's ability to detect biologically meaningful changes past this point. Therefore, to maximize sensitivity for future 3D and 2D studies examining STAT6 activation, I will use a seeding density of approximately 20,000–50,000 macrophages per well to ensure an optimization of signal magnitude, reproducibility, and statistical discrimination for long term co culture modeling.

Comparing the Effect of Plate Reading Position for 3D cell culture on Byonoy Signal Output

Following analysis of bottom read data sets, macrophage signal output was compared between the bottom and top-read data, looking at the absolute difference between the two to determine both optimal macrophages seeding density and the influence of plate position and culture architecture on measured STAT6 activity (**Figure 11D**).

In the bottom-read dataset, statistical discrimination between high-density cultures deteriorated rapidly after day 2. By day 3, essentially all densities $\geq 20k$ cells/well became statistically indistinguishable, and by day 4, nearly every comparison among densities $\geq 5k$ cells/well (i.e., 20k vs. 30k, 30k vs. 50k, and 50k vs. 100k cells/well) was nonsignificant. In contrast, the top-read data retained density-dependent separation longer. On day 2, significant differences were still observed between 20k and 100k cells/well, 20k and 150k cells/well, and 30k and 100k cells/well, while only the highest-density comparisons (50k vs. 100k and 100k vs. 150k) became nonsignificant.

This suggests that the apparent saturation observed in the bottom-read assay is not purely biological. Instead, part of the loss of resolution likely reflects optical limitations

associated with reading through increasingly dense cellular constructs. However, the top read data sets confirmed that denser plating conditions made signal output an unreliable indicator of true bioluminescent signal intensity.

Across all days, both top and bottom measurements demonstrated a clear density-dependent increase in bioluminescence. However, bottom-read values were consistently higher than top-read values across all seeding densities (**Figure 11B,C**). From day 2 onwards, the top read retained significant discrimination among several intermediate and high-density groups that appeared to converge in the bottom-read analysis. For day 2 in the top read, significant differences remained detectable between 20k and 100k cells/well, 20k and 150k cells/well and 30k and 100k cells/well, whereas several of these comparisons were weakened or lost in the bottom-read analysis. Through days 3 and 4, the bottom-read analysis showed extensive convergence among densities ≥ 20 k cells/well, initially suggesting that biological saturation had occurred and that increasing cell number no longer influenced STAT6 signaling. However, the top-read measurements maintained greater separation among intermediate and high-density groups, indicating that the biological response had not completely plateaued. The observation that top-read measurements preserved density-dependent differences while bottom-read measurements did not strongly suggest that the apparent saturation and compression of higher density seeding conditions into similar values observed in the bottom-read assay is at least partially attributable to measurement artifacts rather than exclusively reflecting biological limitations.

Statistical comparisons of the top-minus-bottom differences demonstrated that RLU differences increased as cell density increased. This suggests that optical factors

associated with cell density begin influencing signal detection as macrophage populations expand. If top and bottom readings were simply measuring the same signal equally well, the absolute difference should remain relatively constant across densities. Instead, the difference grows as density increases, indicating that measurement orientation itself is the cause of density-dependent bioluminescence effects.

At low densities (1k–10k cells/well), both reading orientations generally identify similar trends and maintain statistical separation between groups. However, as density increases, bottom readings lose discriminatory power more rapidly than top readings. This divergence suggests that the observed signal in 3D cultures are a mix of both the biological signal and the density-dependent optical bias that increases with macrophage density. Consequently, nonsignificant RLU values at high densities may not be synonymous with M2 polarized cell number, as some of the apparent convergence could reflect limitations in signal detection rather than strictly density-dependent signaling changes

The combined datasets indicate that for both the top and bottom readings, intermediate seeding densities between 20k and 50k offer the highest number of statistically significant pairwise comparisons over long-term cell culture, the lowest optical distortion evidence, and consistent separation in both top and bottom read analysis. While both top and bottom read data sets offer crucial interpretations for 3D culture behavior, bottom read plate measurements provide a higher signal output for all densities across every period,

Implications for 3D Macrophage Cell Culture Set Experiments

When the top-read, bottom-read, and top-bottom difference analyses are considered together, the data supports 20k–50k macrophages/well as the optimal seeding density range. This range consistently produces high STAT6 reporter activity while preserving statistical discrimination between experimental conditions and minimizing density-dependent measurement artifacts.

The bottom-read analysis alone would underestimate the density at which biological saturation occurs because optical attenuation causes premature convergence of high-density groups. The top-read data demonstrate that meaningful biological differences persist beyond 20k–30k cells/well, while the top-bottom difference analysis reveals that measurement bias increases substantially above approximately 50k cells/well.

Therefore, 30k–50k cells/well represents the best compromise between signal strength, dynamic range, and measurement fidelity for future IL-4/STAT6 macrophage reporter studies.

In determining the optimal read position (top or bottom), it's necessary to look at both the statistical separation between values as well as where the bioluminescent signal is generated within the construct. In this study, macrophages were cultured over multiple days, allowing substantial changes in cell morphology, clustering, extracellular matrix deposition, and vertical organization within the well. Under these conditions, the measured signal represents not only STAT6 activation but also the spatial distribution of viable reporter-expressing cells throughout the culture.

Although top reads maintained greater statistical separation among some high-density groups, bottom reads are likely more representative of the biologically relevant signal. Bottom reads offer a higher signal-to-noise ratio with consistently higher RLU values across time points and increased photon capture which is important for improving assay sensitivity and reducing background noise in long term experiments with fluctuating reporter expression. The bottom plate read is closer to the cell-adhered section of the well plate, meaning a shorter photon travel distance which reduces variability caused by media depth, evaporation, condensation or meniscus formation. In addition, deep tissue signal important for 3D culturing is improved with bottom reading, better able to detect signal originating from the interior and lower portions of the construct where many viable macrophages may reside. Top reads in this case may underestimate activity originating deeper within the culture due to increased photon attenuation through the construct and overlying media. In addition, bottom signaling better recapitulated in vivo bioluminescence imaging through reading signal areas from more biologically relevant regions that are experiencing diffusion gradients for oxygen, nutrients and cytokines, therefore better reflecting physiologically relevant STAT6 signaling.

The observation that top reads remained lower than bottom reads throughout the experiment is consistent with the localization of the cells to the bottom of the plate as well as the well configuration, creating more distance between the light source and plate reader with top reads. As cell density increased, the growing discrepancy between top and bottom measurements were most likely a result of increased photon scattering and absorption, greater construct thickness, and increased media path length for top-read detection. Therefore, the top and bottom measurements are sampling different portions

of the optical signal. For long-term 3D cultures, bottom reads may therefore provide a more complete representation of total reporter activity within the construct.

Overall, bottom read bioluminescence measurements may provide substantially higher signal intensity, improved signal-to-noise ratio, greater physiological relevance, and more effective detection of photons originating from deeper regions of the culture.

Further, in a 3D system, the convergence of high-density groups to similar bioluminescence can be a result of both biological saturation and optical effects, making bottom reads the more biologically representative measure of total STAT6 activity over time.

Organoid Co-Culture Conclusions

3D cell culture platforms provide more physiologically relevant platforms for monitoring cell-cell interactions than 2D cell culture. Through increasing physiological ECM dimensionality around the cells, crucial in informing fibrotic mechanisms of ECM dependent inflammation, the proliferation, differentiation, morphology, signaling and mechano-responses of cells better recapitulate bioactivities in disease states (Duval et al., 2017). Spheroids, a type of 3D cell culture platform, can facilitate cell-cell and cell-matrix interactions, provide similar physicochemical in vivo environments and secrete cytokines and chemokines crucial in understanding fibrotic progression. Therefore, they serve as a valuable platform for informing the role of macrophage polarization along the course of pro-inflammatory fibrotic progression. However, studies using spheroid cultures have failed to look at temporal and spatial cues of macrophage polarization, focusing on end-point cell characteristics. This limits analysis of these spheroid co-

cultures to end points of the fibrotic process rather than looking at the progression of fibrosis. However, adding another dimension of cell growth makes incorporating temporal and spatially resolved bioluminescent quantification difficult, necessitating the optimization of platforms to be able to continuously, accurately monitor the STAT6 bioluminescent output along spheroid formation and fibroblast co culture. Therefore, I tested two platforms for monitoring STAT6 activation: 3D Agarose Molds prepared through a Mold Master provided by Microtissues and Corning 96-Well, Ultra-Low Binding, U-Shaped-Bottom Microplate for spheroid formation. The 3D Agarose Molds failed to produce observable bioluminescent signal output, likely due to the thickness of the agarose mold, inability to spatially discern spheroid signal output, and cell overgrowth expanding out of the mold onto the well bottom. Further, the inability to remove spheroids from the mold for continued culture made real time, continued bioluminescent signal monitoring difficult. Therefore, Corning 96-Well, Ultra-Low Binding, U-Shaped-Bottom Microplates for high throughput spheroid formation was optimized to provide real time, temporal data for BTS platforms utilizing the Byonoy Luminescence 96 plate reader following activation of STAT6 in RAW 264.7 STAT6-CeNL macrophages. Higher RLU intensity outputs from the bottom plate reader position vs top position from the Byonoy signified localization of macrophages to the bottom of the well plate and provided more physiologically relevant measurements due to omitting media distortion effects that may result from top-down plate reads. Further, over a long-term culture of macrophages after inducing STAT6 activity, wells seeded at 20k-50k cells/ well provided concentration-dependent signal increases throughout the course of the experiment, remaining statistically significant with limited signal convergence seen

as was observed by higher concentrations. Overall, 3D cell culture with 96-Well, Ultra-Low Binding, U-Shaped-Bottom Microplates were able to provide temporally and spatially resolved signaling of STAT6 activation in macrophages over long term spheroid formation. Optimization of a 3D cell culture platform, plate reader position and initial macrophage seeding density will inform future experiments involving 3D spheroid formation to ensure reliable, accurate bioluminescent signal output.

Chapter 5: The Effect of Nanoplastics on STAT6 Signaling in Macrophages

Significance

The goal is to uncover the effect of polystyrene (PS) and polypropylene (PP) nanoplastics (NP) on macrophage polarization in both fibrotic and non-fibrotic settings using Bioluminescence Temporal Spectroscopy. We aim to demonstrate how the addition of plastics impacts STAT6 signaling a feature of foreign body response signaling. We hypothesize that adding PS NPs over time will increase STAT6 signaling in a concentration-dependent response.

Background

Nanoplastics (NPs), particles with sizes less than 1000 nm, are considered an increasing ecological problem with serious human health effects due to the increasing use of plastics in everyday products and life (Kreuter, 1996). An average plastic concentration of 1.6 $\mu\text{g/mL}$ has been found in the blood, composed largely of styrene and polystyrene (PS), which are typically used for food packaging (M. Y. Adler et al., 2024a; Leslie et al., 2022). Despite such an increasingly pressing issue, the longitudinal, real-world exposure effects of NPs are still poorly understood (Fan & Ha, 2025a).

NPs and microplastics (MPs), particles ranging from 1-1000 μM , can enter the body through inhalation, skin contact and ingestion through contaminated food and water (Fan & Ha, 2025b). However, internalization of NPs pose a greater threat than MPs,

with concentrations in the environment 10 to the 14 higher than MPs, resulting in higher bioreactivity (Chen et al., 2023; Enfrin et al., 2020). NPs are toxicologically more relevant than MPs, resulting in increased inflammation and inflammatory signaling, such as cytokine secretion and inflammatory cell infiltration (Chen et al., 2023). Therefore, particles under 1 μ M composed of polystyrene, detected at large rates in the human blood, are used in this study to best recapitulate physiological relevant conditions.

Macrophages are activated by exposure to foreign particulate matter such as NPs, resulting in macrophage subpopulations with altered phenotypes (Collin-Faure et al., 2023; Rudolph et al., 2021). Previous studies have shown that macrophages internalize PS MNPS, with smaller sized particles causing higher internalization, resulting in altered cell metabolism (Bruinink et al., 2015; Collin-Faure et al., 2023). However, toxicity to PS-NPs has been observed in macrophages depending on particle size, concentration and exposure duration (M. Xu et al., 2019). Cytotoxicity to macrophages was found to increase as particle size decreased (Wolff et al., 2023). Increasing incubation time of PS-NPs was shown to increase apoptotic cell percentage (M. Xu et al., 2019). Overall, this paints a picture of smaller particles, higher concentrations and longer exposure times to MNPs resulting in increased cytotoxicity of macrophages.

Plastic Particles Effect on Macrophage Polarization

The impact of plastic particles, specifically PS NPs, on macrophage polarization are varied. PS NPs have been shown to upregulate phosphorylated ERK and p65 in a dose dependent manner, activating the MAPK and NP- κ B pathways along with increasing reactive oxygen species (Q. Hu et al., 2021; Liu et al., 2020). However, several studies

have suggested that PS MNPs are unable to activate either M1 or M2 polarization states. PS beads ranging in size from 0.5 to 3 μM were unable to induce polarization of macrophages, despite featuring uptake by macrophages (M. Y. Adler et al., 2024b). Another study incubating MPs ranging from 1 to 10 μM with macrophages showed comparable levels of STAT6 and STAT1 in M2 and M1 macrophages as cells not previously exposed to PS MPs, suggesting that MPs have no effect on pro-inflammatory or anti-inflammatory signaling (Stock et al., 2019).

Studies have mixed reviews regarding the ability of PS-NPs to induce pro-inflammatory or anti-inflammatory responses. One study found that PS-NPs 25 and 70 nm diameter upregulated pro-inflammatory cytokines such as IL-8, NF- κB , and TNF α as well as activating several pro-apoptotic proteins (M. Xu et al., 2019). Further, phagocytosis of PS MPs by macrophages activated glycolytic pathways, as detected by the cell-surface markers CD80 and CD86, hallmarks of pro-inflammatory macrophages (Merkley et al., 2022). However, this study was limited to a 16-hour incubation period, when pro-inflammatory responses are known to be high, potentially excluding later M2 polarization phenotypes. Concentration-dependent activation of M1 cell markers and cytokines in macrophages was observed by Jiang et al when incubating 50 and 500 nm PS NPs with macrophages (W. Jiang et al., 2024). However, data were collected as an endpoint measurement, omitting potential M1/M2 transition stages.

Other studies observe upregulation of M2 polarization markers upon PS-NP/PS-MPs activation. The upregulation of TGF- β and IL-10 was observed by histopathological analysis of PS-MPs ranging from 10-50 μM incubated with macrophages (Y. Lee et al., 2025). In pregnant mice, 10 μM PS-MP exposed macrophages in the peripheral blood,

spleen and placenta shifted to a M2 polarized phenotype, detected by an increase in the CD206 cell surface marker (J. Hu et al., 2021). Notably, this study was run over a longer 11.5-day time course following PS-NP injection at days 5.5 and 7.5. This polarization of macrophages to an M2 phenotype was confirmed in mice through an 18 week time course experiment featuring administration of 1 μ M PS-MP (W. Xu et al., 2024). Finally, 50 nm PS-NPs incubated with trout macrophages upregulated the anti-inflammatory cytokine IL10 and M2-associated proteins MMP9 and Cathepsin D, while failing to upregulate the pro-inflammatory cytokines IL1 β IL12, and TNF α (Brandts et al., 2023).

Other studies postulate a potential dual inflammatory effect of PS-NPs. Wolf et al observed decreases in macrophage pro-inflammatory cell markers CD80 following 24- or 72-hour incubation with either 50, 200 and 1000 nm PS-NPs. Interestingly, 200 and 1000nm PS-NPs increased the production of the anti-inflammatory IL-10 cytokine at the same time that IL-6 and other pro-inflammatory cytokines were released (Wolff et al., 2023). This suggests a dual anti- and pro-inflammatory axis upon PS-MNP exposure, further underscoring the need for real-time measurements of M2 polarization.

Overall, the effects of PS-NPs on macrophage polarization are unclear due to contradictory conclusions by a host of studies. This can be due to the use of proteomic or transcriptomic approaches that are based in end-point measurements, such as flow cytometry or RNA-seq. As macrophages are complex, dynamic cells that transition between differentiated and polarized M2 and M1 states due to local environmental cues and signaling, these studies are unable to capture the true scope of their dynamic processes and responses, relevant in diseases and inflammatory contexts. Temporal measurements, monitoring polarization states time point over a longer incubation period

would add to analysis based on end-point parameters. Further, the size, along with the type and functional group, of MP/NPs has been found to impact macrophages, fibroblasts, relevant in fibrosis, and overall particle distribution in vastly different ways (Olivier et al., 2003). Many previous studies, looking at larger PS particles as well as analysis focusing on short term, end-point measurements, lack the temporality and complexity of true dynamic macrophage environment interactions, limiting conclusions and creating varied results. Therefore, the use of long-term, temporal data through bioluminescent imaging of STAT6, a M2 polarization marker, offers insight into the scope of true macrophage polarization behavior.

Characterization of PS NPs by DLS

Dragon Green Fluorescent Carboxyl Polystyrene Microspheres were obtained from Bangs Labs at 1 mL aqueous suspension at a size of 0.040-0.060 μM . To confirm their size and determine their spectral output, Dynamic Light Scattering (DLS) measurements were performed on 3 bead dilutions. It is important to verify both the particle size and whether the PS-NPs remained monodisperse in suspension or aggregated. As macrophage uptake, intracellular trafficking and immunomodulatory effects are highly influenced by particle size, the DLS measurements provide crucial context for interpretation of future macrophage polarization responses.

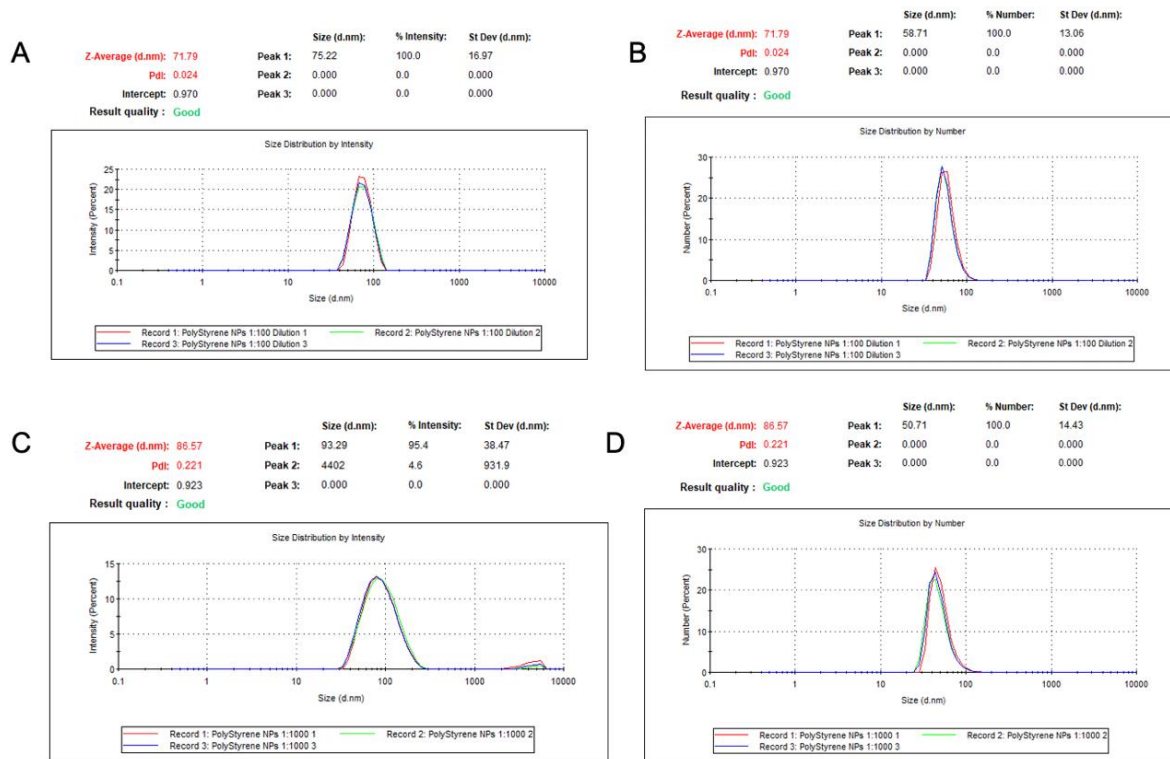


Figure 12 DLS Measurements with Size Distribution by Intensity and Number for 1:100 and 1:10,000 dilutions

The size distribution of the PS-NPs were observed with Dynamic Light Scattering (DLS). Graphed are the size distributions by A) intensity for a 1:100 dilution, B) number for a 1:100 dilution, C) intensity for a 1:10,000 dilution and D) number for a 1:10000 dilution.

The 1:100 and 1:10,000 dilutions were able to provide clear signal for analysis, while the 1:1,000,000 dilution was too dilute to provide a substantive reading. Looking at **Figure 12**, the 1:100 dilution exhibited a highly reproducible particle size distribution across all three replicates, with the dominant peak centered between approximately 50–60 nm. The maximum intensity occurred near 50.7–58.8 nm, where all three replicates showed similar distributions, indicating high measurement reproducibility. The narrow distribution with most signal contained between approximately 38 and 90 nm and very little intensity above 120 nm shows high monodispersity / signal confinement to the predicted range. The 1:10,000 dilution produced a nearly identical primary peak, also

centered around 44–58 nm, with the highest intensity occurring between 43.8 and 50.7 nm. Measurements were repeated three times to ensure replicability. Replicate agreement remained strong, indicating that dilution did not substantially alter particle size distribution, even while decreasing PS-NP average size.

There is no large secondary peak in the micron range for either of the two dilutions, highlighting that even with the intense sensitivity of DLS measurements, the micron-sized populations were negligible in comparison to the dominant 50-60 nm population. Therefore, the PS-NP can be considered largely monodisperse with minimal aggregation, important for ensuring that changes in STAT6 bioluminescence intensity was due to PS-NP exposure rather than artifacts from particle aggregation or altered size distributions. The similarity between the two dilution conditions confirms the advertised size range and distribution of the particles even when diluted, thereby ensuring that there is not a concentration-dependent artifact that could interfere with STAT6 activation behavior or signaling dynamics.

Serial Dilution to Determine PS-NP Fluorescence Activity

A serial dilution was necessary to confirm that fluorescent PS-NP stock could be reproducibly diluted while maintaining detectable particle concentration across many concentrations. Importantly, performing a serial dilution was necessary to correlate PS-NP concentration readings with fluorescence output. The stock solution of PS-NPs at $1\text{E}14$ was compared to 1:100, 1:1,000 and 1:10,000 dilutions with the 1:100,000 dilution omitted to low fluorescence output. Looking at **Figure 13A**, each successive dilution was able to provide a statistically significant decrease in fluorescence intensity

($p < 0.0001$), confirming that the serial dilution produced predictable reductions in detectable particle concentrations. However, the 1:1000 and 1:10,000 dilutions were not statistically significant ($p = 0.148$), suggesting that these dilutions were too close to provide discriminatory differences in measurement intensity. Therefore, only one of these dilutions was included for the 4-day bioluminescent time course experiment, with the concentrations selected (100, 10,000 and 100,000) spanning approximately 3 orders of magnitude and including a low, middle and high exposure group. Taken together with the DLS characterization demonstrating stable nanoparticle size distributions, support the selection of low, intermediate and high PS-NP exposure conditions for the macrophage coculture experiments. Further, the macrophage and PS-NP co culture can be interpreted as biologically meaningful responses to increasing nanoparticle burden rather than artifacts arising from nanoparticle instability or inconsistent dilution preparation.

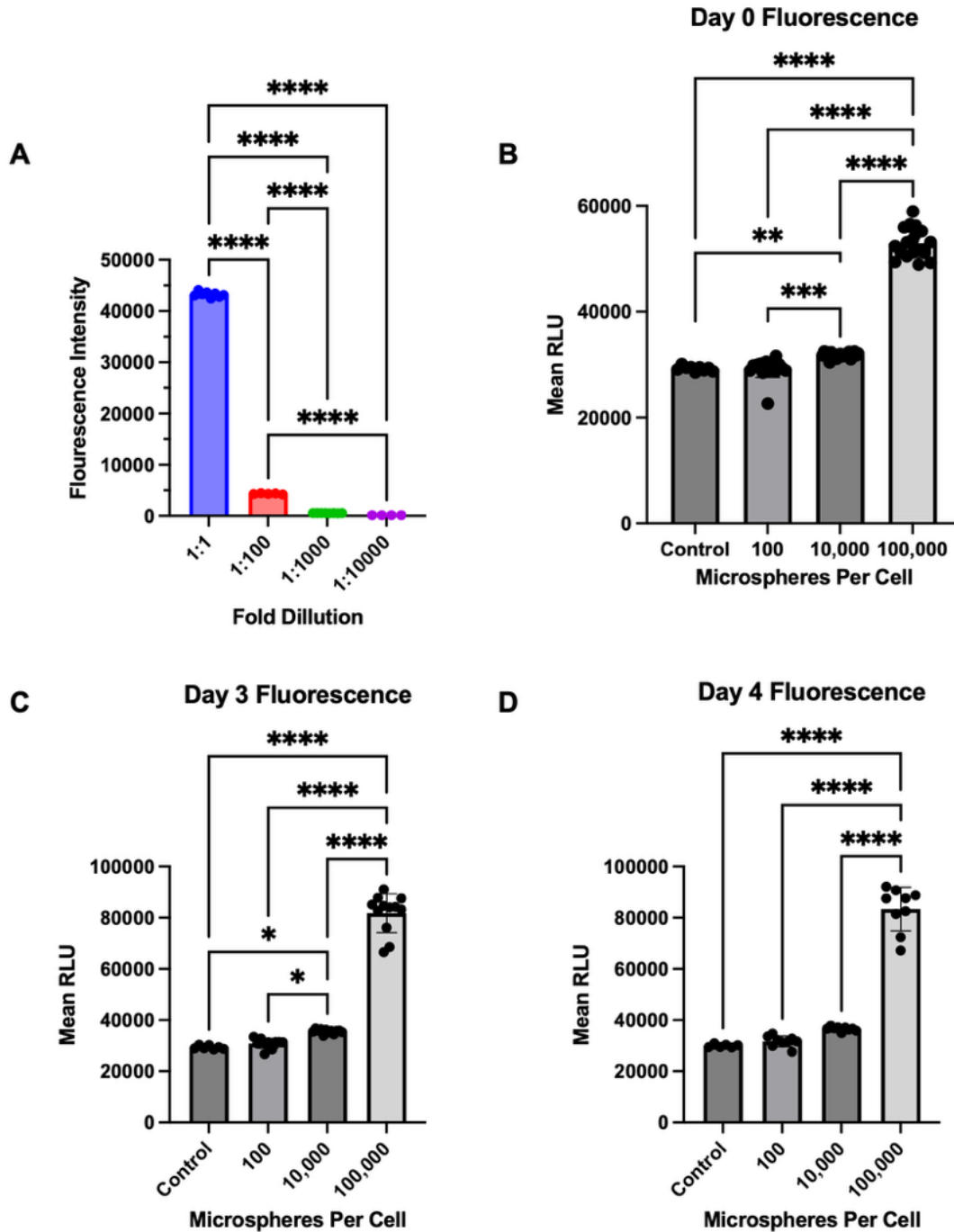


Figure 13 Fluorescence Output of RAW 264.7 STAT6 Macrophages with PS-NPs Over Time

Fluorescence Measurements were performed on the Synergy h1 plate reader to monitor the fluorescence of the *Fluorescent Carboxyl Polystyrene Microspheres, Dragon Green - 0.05 μ m, PS-COOH-(480,520)*. A) Fluorescence read outs of microspheres diluted with distilled water at 1:1, 1:100, 1:1000 and 1:10,000 concentrations. 264.7 RAW STAT6 Macrophages were seeded at 30,000 cells/ well, allowed to rest for one day before 3 concentrations of microspheres were added. Fluorescence measurements were taken on B) Day 0 C) Day 3 and D) Day 4. Sample Size. N= 9, N= 7, N=6 for Control Day 0, 3, and 4, respectively. N= 21, 12, and 9 for Experimental Groups Day 0, 3, and 4, respectively. **Control = only RAW 264.7 STAT6 cells. Statistical Significance: Ordinary one-way Anova**

and TukeyPost-hoc test with Multiple Comparisons. * $p < 0.033$, ** $p < 0.002$, *** $p < 0.0002$, **** $p < 0.0001$, ns (non-significant)

PS-NP and Macrophage Incubation Experimental Findings

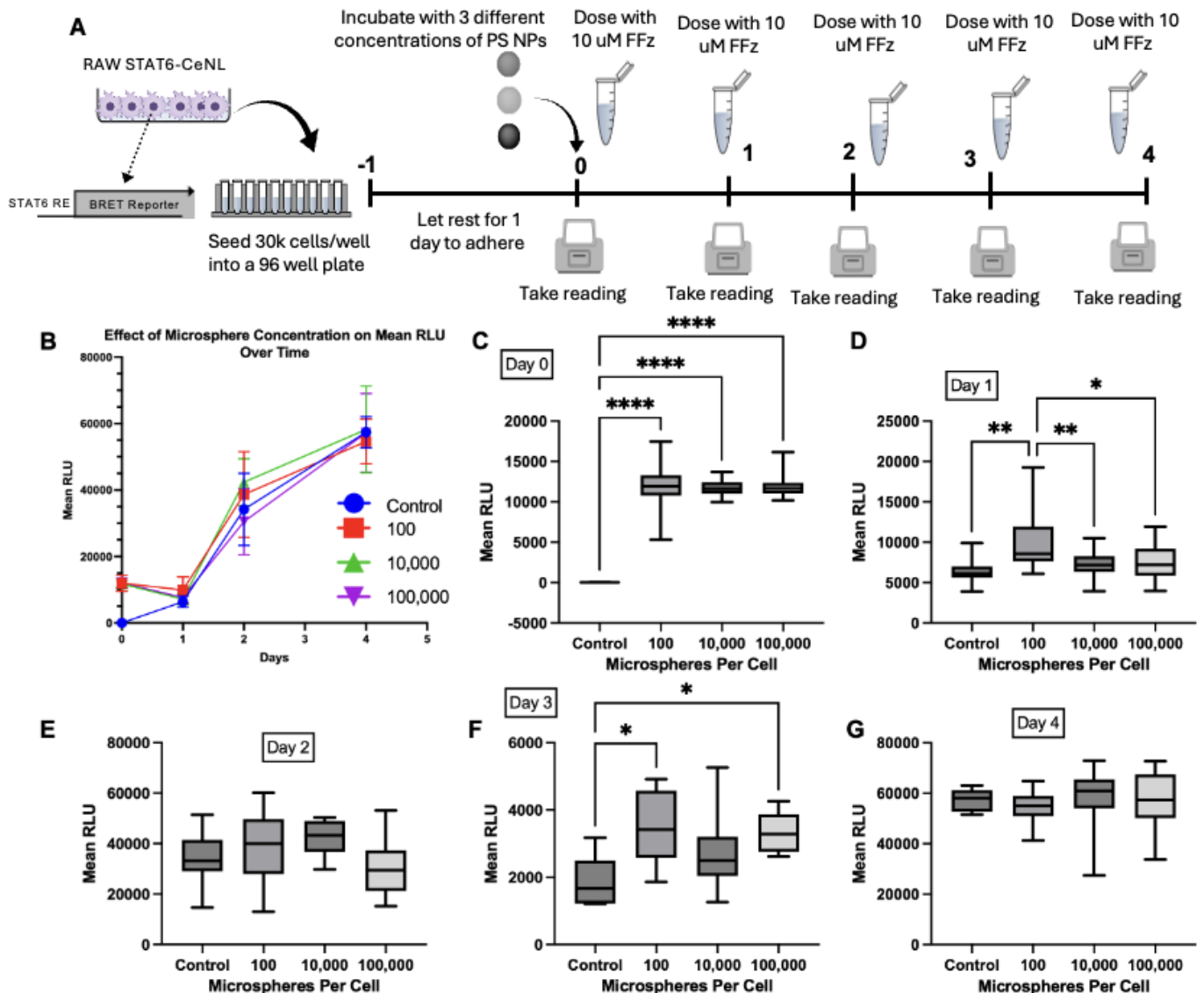


Figure 14 RAW 264.7 STAT6 Macrophage Incubation with PS-NPs Over Time

A) Experimental schematic for the incubation of PS-NPs with RAW 264.7 STAT6-CeNL macrophages. Briefly, RAW 264.7 STAT6-CeNL Macrophages were seeded at 30k/well in a 96 well plate and allowed to adhere for 1 day. At Day 0, 3 different concentrations of *Fluorescent Carboxyl Polystyrene Microspheres* (PS-NPs), *Dragon Green - 0.05 μ m, PS-COOH-(480,520)* in DMEM high glucose media were added to the wells. Bioluminescence measurements were taken on the Luminescence 96 Byonoy (BiotekPlate reader) over the course of 4 days and graphed according to: B) STAT6 Mean RLU for the three different concentrations of PS-NPs vs control over the 4 day time course C) Day 0, D) Day 1, E) Day 2, F) Day 3, G) Day 4. Sample Size. N= 9, N= 8, N=8, N=6, N=6 for Control Day 0, 1, 2, 3, and 4,

respectively. N= 21, 18, and 9 for Experimental Groups Day 0, 1, and 2- 4, respectively. **Control = only RAW 264.7 STAT6 cells. Statistical Significance: Ordinary one-way Anova and Tukey Post-hoc test with Multiple Comparisons. *p<0.033, **p<0.002, ***p<0.0002, ****p<0.0001, ns (non-significant)**

A 4-day time course experiment was performed to measure the effect of PS-NP culture at three different concentrations with RAW 264.7 STAT6 CeNL macrophages to determine the effect of microplastic exposure on STAT6-associated transcription **(Figure 14A)**. Macrophages were seeded at 30,000 cells per well in 100 μ L of high-glucose DMEM media. Cells were allowed to rest for 24 hours to promote adherence to the plate bottom before being incubated with three different Polystyrene (PS)- Nanoparticle (NP) concentrations (1:100, 1:10,000, and 1:100,000). Cells were dosed with 10 μ M FFz immediately to catalyze the bioluminescence reaction and a day 0 measurement on the Byonoy plate reader was taken along with a fluorescence measurement and a picture on the Revolve Echo microscope. Every 24 hours following, cells were dosed with 10 μ M FFz, and a bioluminescence measurement and fluorescence measurement were taken. Importantly, macrophages were not activated with IL4 prior to PS-NP exposure; therefore, the bioluminescence STAT6 behavior reflects basal STAT6-dependent transcriptional activity modulated by PS-NP exposure rather than cytokine-driven alternative macrophage activation. Fluorescence measurements were taken for day 0, day 3 and day 4, highlighting the stable output of PS-NP fluorescence and presence in the sample across the four day time period **(Figure 13B-D)**.

Figure 14B depicts RLU measurements for control (untreated macrophages cultured in DMEM media), 100, 10,000 and 100,000 bead conditions across days 0-4. Day 0 is the baseline and is not interpreted as a biological response timepoint. The effect of bead

exposure on STAT6 signaling in macrophages graphed together over days. Notably, while STAT6 activity increases from days 1-3, there appears to be little difference across the various PS-NP concentrations and between these concentrations and control measurements. Therefore, while STAT6 activity may be increasing, it seems unlikely to be due to exposure to PS-NPs. However, looking at data day by day, there emerges a potential effect on PS-NPs within 24-48 hours on STAT6 activity, while not dose-dependent.

At day 1 while there wasn't a clear, PS-NP concentration-dependent response, there were small, statistically significant differences between the 100 PS-NP concentration and the control, 10,000, and 100,000 PS-NP conditions (**Figure 14D**). However, there are no significant differences between any other conditions at this time point. Significant effects between conditions seem to disappear at day 2 even as STAT6 activation increases (**Figure 14E**). However, at day 3 significant differences are seen between control and 100 and control and 100,000 microsphere per well conditions, suggesting that the lowest and highest bead exposures were able to induce STAT6 transcriptional activation. However, as these differences are seen only in day 3 with day 2 and day 4 showing no significant differences across any condition, the effects of PS-NPs on STAT6 activation may be seen as transient rather than sustained.

The temporal response pattern suggests that PS-NP exposure exerts its greatest influence on macrophage STAT6 activity during the first 24–48 hours following exposure. Concentration-dependent differences were most apparent during the early stages of coculture and diminished at later time points, indicating that macrophages may undergo an adaptive response to nanoparticle exposure. The convergence of

reporter activity by day 4 suggests that the effects of PS-NPs on STAT6 signaling are transient rather than sustained. These findings support the hypothesis that PS-NPs can modulate early macrophage signaling events but do not provide evidence for persistent STAT6 activation or stable M2 polarization. Instead, the data are consistent with acute nanoparticle-induced perturbation followed by cellular adaptation and restoration of signaling homeostasis. However, an alternative theory based on increasing cell density between each condition is suggested through **Figure 15**.

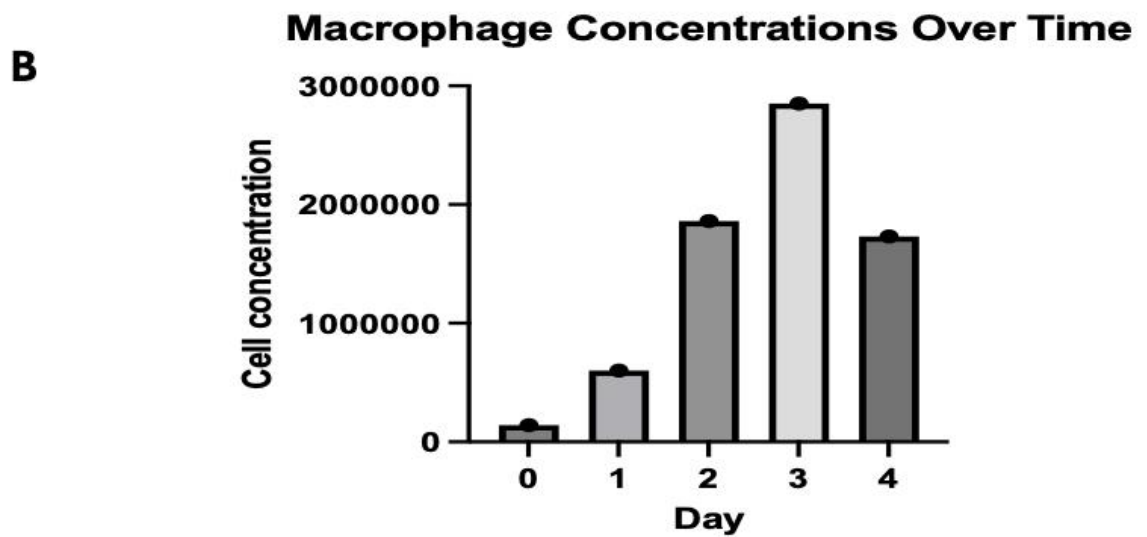
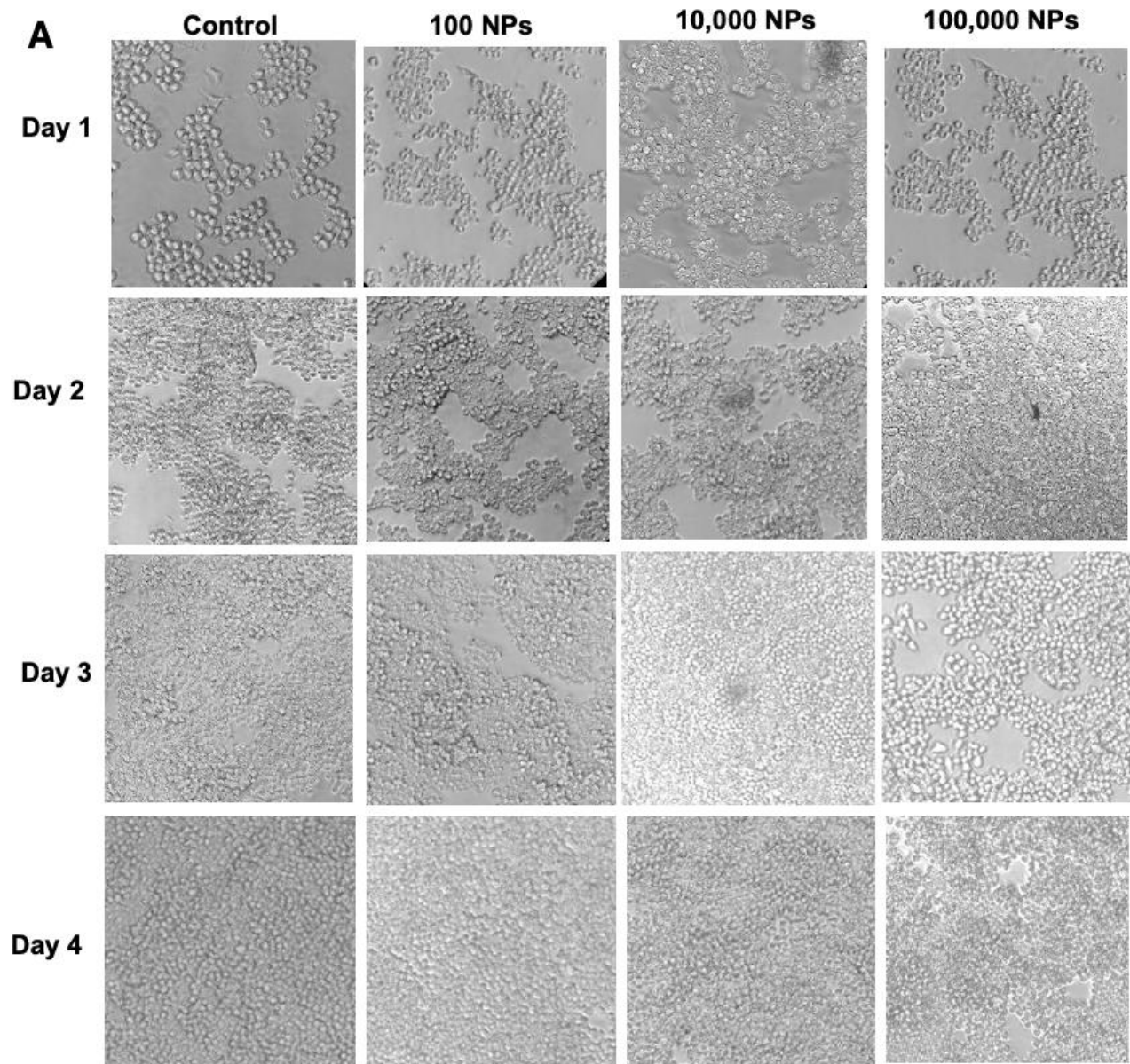


Figure 15 2D Macrophage and PS-NP Co Culture Cell Confluency Over Time

RAW 264.7 STAT6-CeNL Macrophages were seeded at 30k/well in a 96 well plate and allowed to adhere for 1 day. At Day 0, 3 different concentrations of *Fluorescent Carboxyl Polystyrene Microspheres* (PS-NPs), *Dragon Green - 0.05 μ m, PS-COOH-(480,520)* in DMEM high glucose media were added to the wells. A) Images were taken on the Revolve Echo Microscope on Day 1-4 for Control, 100 NPs per well, 10,000 NP per well and 100,000 NP per well. B) Cell confluency of the control condition was measured over 4 days. Briefly, cells were trypsinized to remove from well plate, then spun down and resuspended in DMEM high glucose media and counted.

Images of each microsphere condition (control, 100 per well, 10,000 per well and 100,000 per well) were taken at day 0 and each subsequent day throughout the course of the experiment (**Figure 15A**). Cell confluency of the control condition showed increases in cell proliferation from day 0 through day 3 of the time course experiment with decreases in cell growth seen at day 4 (**Figure 15B**). This suggests that cell growth and proliferation occurred from day 0-day 3, increasing the density within each well at least for the control condition that could have resulted in a higher bioluminescent signal from a growing macrophage population containing the STAT6 RE that could be responsive to microsphere activation.

Across days and conditions, images depict varying cell densities, potentially a result of microsphere incubation at different ratios interacting with the RAW 264.7 STAT6 macrophages. For all conditions, cell density increases from day 1 onwards, with little differences seen in images between day 3 and 4. On day 1, the 10,000 NP/well condition looks to have a higher cell density than the other three conditions while on day 2, the 100,000 NP/well condition seemed to have the higher cell density. Day 3 featured marked increases in the cell density of the control, 100 and 10,000 NP/well conditions but seemed to feature a decrease in cell density of the 100,000 NP/well condition. By day 4, all conditions looked to have widespread cell adherence, with macrophages coating the bottom of the wells. Overall, cell density is clearly changing over time, increasing day by day for each condition. However, cell density seems to vary between

NP concentration conditions as well, suggesting that the nanoplastics might influence cell proliferation and cell health. Particularly, macrophages between conditions might have altered phenotypes reflective of cell health.

Altogether, this necessitates normalization between condition based on cell number to ensure that the degree of cell bioluminescent output is reflective of normalized STAT6 activation rather than cell number, which varies between condition over time. Further, a comparison of other phenotypic cell health parameters and polarization markers between experimental conditions and controls is necessary to determine the effects of PS-NPs on macrophage health, phenotype and function. Flow cytometry to characterize macrophages according to polarization and assess cell health in addition to fluorometric DNA quantification with DAPI staining would add to assessment parameters for PS-NP and macrophage incubation. In addition, cytokine and metabolite assays to determine macrophage secretion profiles from PS-NP incubation would help to determine what pathway and macrophages responses were being induced. Overall, quantification of the cell density between cell conditions and days is needed to normalize the bioluminescent measurements by macrophage cell number and therefore truly compare STAT6 activation between days and conditions. Further, parameters to assess macrophage cell health and excretion profiles would help to elucidate the activated pathways and macrophage responses due to PS-NP incubation.

As culture with macrophage and PS-NPs failed to induce statistically significant STAT6 activation between conditions, I wanted to explore different pathways of macrophage activation with bioluminescence. Therefore, I sought to activate the STAT6 pathway with IL4 to induce detectable bioluminescent signal before incubation with different

microsphere concentrations. From there, the anti-inflammatory STAT6 pathway activity could be monitored following microsphere exposure. As I predicted that PS-NPs induced a pro-inflammatory response, I expected STAT6 activity to follow concentration dependent decreases, with control conditions experiencing continually rising STAT6 activity. Overall, microsphere and macrophage incubation alone failed to induce long-term STAT6 activation differences between conditions and between conditions and controls, suggesting microspheres activated macrophages by different pathways.

IL4 Priming of Macrophage: Microsphere Co-cultures

Upon stimulation with activating substances, such as IL-4, STAT6 rapidly binds to target gene promoters, producing observable transcriptional changes within 1 to 3 hours. However, broader alternative macrophage M2 polarization pathways take between 6 to 24 hours to develop (Czimmerer et al., 2018). Furthermore, temporal analysis of IL4 activation and STAT6 bioluminescence expression showed a peak in STAT6 activity between 1 and 2 days after IL4 exposure. Taken together, this suggests that STAT6 activity monitored by bioluminescence experiences delayed activation, with peak activity expected between 1 to 2 days of exposure. Notably, IL4-mediated STAT6 activation experiences a sharp increase in activity until peak activation, upon which STAT6 activation rapidly declines. Therefore, continuous monitoring of STAT6 activation following IL4 exposure is critical for finding peak STAT6 activation.

While IL4 administration was expected to increase STAT6 bioluminescence within a 24-hour time period, multiple trials run failed to induce a statistically significant STAT6 signal compared to controls over this time period. This could be due to the age of IL4

aliquots or potential cell contamination. Overall, testing the effects of initial IL4 incubation prior to microsphere addition on STAT6 signaling would provide crucial evidence for the inflammatory pathway PS-NPs are activating. A better knowledge of the role of PS-NPs in exacerbating inflammation and in fibrotic pathways would inform therapeutic interventions as the rate of nano and microplastics in the body rises.

Section 5 Conclusion

Overall, even as rates of microplastics and nanoplastics in the bloodstream are increasing, their effects on both the inflammatory pathway and in fibrosis remain largely unexplored. Further, their effect on macrophages remains conflicted, with articles observing differences in M1 vs M2 activation profiles depending on particle size, concentration and type. Their effects on macrophages and in diseases, especially fibrosis, are crucial towards considerations in future therapeutics and changing treatments due to their potential effects in exacerbating the body's inflammatory response. I sought to explore the effect of different concentrations of PS-NPs on RAW 264.7 STAT6 macrophages over time, tracking STAT6 bioluminescent output as a proxy for M2 macrophage polarization over time. Incubation of macrophages and PS-NPs failed to induce long term STAT6 activation over a four-day time course relative to controls. This was not due to failure of PS-NP to remain in culture, as nanoparticle fluorescence remained constant between conditions over time. However, statistically significant differences in bioluminescent output was seen on day 1 and 3, highlighting increases STAT6 activation between the 100 NP/well and control, 10,000 and 100,000 conditions for day 1 and increased STAT6 activation between the 100 and 100,000 PS-NP conditions and the control for day 3. These differences suggest that STAT6 is being

activated at certain points upon PS-NP incubation for select concentrations, but failure to sustain long term STAT6 activation limits definitive statements macrophage profiles upon exposure to nano plastic beads. Images taken over the 4 days depict changes in macrophage cell density between conditions and across time, necessitating quantitative cell density and cell health parameters between conditions and over time to understand the true effect of microspheres on STAT6 activity. The increase in cell confluency through day 3 for the control condition highlights cell growth over time that could vary between condition and therefore requires normalization dependent on cell number across conditions. Altogether, this suggests an alternative pathway to macrophage activation in response to PS-NP incubation, although further quantitative parameters are needed to further assess this response.

Activation of RAW 264.7 STAT6 Macrophages by IL4 prior to microsphere incubation would induce the M2-IL4 STAT6 pathway. Therefore, the effect of nanoparticles on this STAT6 pathway could be assessed following incubation, denoting decreases or increases in STAT6 activity depending on whether pro or anti-inflammatory pathways are being induced. However, failure of IL4 to activate the STAT6 pathway in multiple trials suggests either contaminated IL4 or RAW 264.7 STAT6-CeNL macrophages that requires alternative culturing conditions for future experiments.

Conclusions/ Next Steps

The immune microenvironment involves a complex interplay of diverse cells, tissues, and organs that respond to a host of signals in their microenvironment. Macrophages serve as the first responders to invading pathogens, crucial in the innate immune system and responsible for triggering long term immune responses. Fibrosis, a result of excessive inflammation leading to the buildup of connective tissue, can occur during organ failure or in response to foreign body implants. Macrophages are critically implicated in this process, due their high plasticity and ability to be polarized along a spectrum into anti and pro inflammatory phenotypes. The STAT6 pathway is implicated in the inflammatory fibrotic process through polarization of macrophages to their M2 anti-inflammatory phenotype. The ability to track macrophage inflammatory states throughout the course of fibrosis using macrophage immune cell biosensors tied based on the STAT6 reporter element would provide temporal and spatially resolved cues throughout the fibrotic process. These insights would accelerate therapeutic and disease interventions for fibrosis by providing real time as opposed to end-point measurements along the fibrotic process. Experiments performed have led to the optimization of the bioluminescence temporal spectroscopy (BTS) platform for long term, repeatable measurements of STAT6 activation in macrophages in response to IL4 and fibroblast cells and activated media. Further, this immune cell biosensor platform in macrophages can be applied to 3D cell culture to better recapitulate the fibrotic microenvironment. Including PS-NPs in culture with macrophages to track STAT6 activation highlights another component of disease monitoring that must be included with growing plastic concentration in the blood stream. Incorporating these plastic

particles in incubation with macrophages and fibroblasts within 3D cell culture would create the most relevant platform for studying the fibrotic microenvironment. Altogether, my work creates an optimized pipeline for BTS measurements to provide quantifiable temporal and spatially resolved outputs of STAT6 activation as a proxy for macrophage polarization throughout the fibrotic process. This platform can be employed many different microenvironmental conditions that resemble to complex fibrotic cellular microenvironment. Further, this immune cell biosensor platform based in BTS can be applied to other diseases, changed to be based on different reporter elements and applied at a high-throughput scale to provide personalized medical treatment for disease treatment and prevention. An optimized platform for BTS can be applied to not just fibrosis, but any disease condition to add temporal and spatially resolved signaling lacking in current end-point analysis.

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