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The Nitroreductase System of Inducible Targeted Ablation Facilitates Cell-specific Regenerative Studies in Zebrafish

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Abstract

At the turn of the 20th century, classical regenerative biology – the study of organismal/tissue/limb regeneration in animals such as crayfish, snails, and planaria – garnered much attention. However, scientific luminaries such as Thomas Hunt Morgan eventually turned to other fields after concluding that inquiries into regenerative mechanisms were largely intractable beyond observational intrigues. The field of regeneration has enjoyed a resurgence in research activity at the turn of the 21st century, in large part due to “the promise” of cultured stem cells regarding reparative therapeutic approaches. Additionally, genomics-based methods that allow sophisticated genetic/molecular manipulations to be carried out in nearly any species have extended organismal regenerative biology well beyond observational limits. Throughout its history, complex paradigms such as limb regeneration – involving multiple tissue/cell types, thus, potentially multiple stem cell subtypes – have predominated the regenerative biology field. Conversely, *cellular* regeneration – the replacement of specific cell types – has been studied from only a few perspectives (predominantly muscle and mechanosensory hair cells). Yet, many of the degenerative diseases that regenerative biology hopes to address involve the loss of individual cell types; thus, a primary emphasis of the embryonic/induced stem cell field is defining culture conditions which promote cell-specific differentiation. Here we will discuss recent methodological approaches that promote the study of cell-specific regeneration. Such paradigms can reveal how the differentiation of specific cell types and regenerative potential of discrete stem cell niches are regulated. In particular, we will focus on how the nitroreductase (NTR) system of inducible targeted cell ablation facilitates: 1) large-scale genetic and chemical screens for identifying factors that regulate regeneration and, 2) *in vivo* time-lapse imaging experiments aimed at investigating regenerative processes more directly. Combining powerful screening and imaging technologies with targeted ablation systems can expand our understanding of how individual stem cell niches are regulated. The former approach promotes the development of therapies aimed at enhancing regenerative potentials in humans, the latter facilitates investigation of phenomena that are otherwise difficult to resolve, such as the role of cellular transdifferentiation or the innate immune system in regenerative paradigms.

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Conflict of interest statement: Dr. Mumm holds several patents regarding use of the NTR system in zebrafish and provides consulting services to a biotechnology company, Luminomics, that he founded to pursue NTR-based degenerative disease modeling.

1 Tissue Regeneration in Zebrafish

Zebrafish, like many members of the ray-finned fishes (teleosts), have an innate capacity to regenerate tissues (e.g., fins, heart, eye). Combined with amenability to forward genetic screens and reverse genetic techniques (e.g., morpholino ‘knock down’), zebrafish are providing key insights into regenerative processes. For instance, analysis of caudal fin regeneration has provided insight into mechanisms regulating blastema formation, tissue outgrowth, and patterning [1]. Similarly, factors regulating blood vessel branching morphogenesis in regenerating fins were identified through a screen for temperature-sensitive mutants [2]. While fin regeneration can be viewed as analogous to limb regeneration, it is the capacity to regenerate heart tissue that firmly set the zebrafish model system on the world stage [3].

In the years since this seminal report, researchers have succeeded in revealing mechanisms regulating heart regeneration. One intriguing finding is that heart muscle regeneration in zebrafish does not require a permanent resident stem cell population. Instead, mature muscle cells dedifferentiate to a stem/progenitor state, proliferate, and their progeny replace damaged cardiac muscle [4]. The British Heart Foundation intends to invest millions to determine if this ability is translational to damaged human heart tissue.

Zebrafish have also been shown to regenerate retinal tissue through a similar mechanism [5]. Following injury, Müller glia cells dedifferentiate to a stem-like state and proliferate to replace lost retinal cells. Importantly, this capacity to repair neural tissue damage is not limited to the eye. Recently, an Australian group demonstrated that zebrafish utilize fibroblast growth factor signaling to repair spinal cord injuries without scarring [6]. The absence of scarring is thought to underlie an enhanced capacity for nervous system repair in zebrafish. The primary emphasis of regenerative studies in the nervous system, however, is on cellular repair (i.e., axonal regeneration) as opposed to whole cell replacement. Despite significance for many degenerative diseases – where significant cell loss often precedes disease detection, thus regeneration stands as the only means to regain lost function – the study of cell-specific regeneration has been far less common than investigations of tissue regeneration and cellular repair.

2 Cell-specific Ablation and Regeneration in Zebrafish

Investigations of mechanosensory hair cell loss and replacement within neuromasts of the lateral line (a peripheral linearly arrayed system of sensory organs) initially determined that the regenerative capacity of zebrafish extends to the level of individual cell types [7]. These studies were facilitated by aminoglycosides (i.e., antibiotics) which are toxic to hair cells, thus providing a simple chemically-induced cell ablation methodology. Moreover, fluorescent dyes that quickly and reproducibly label hair cells (e.g., FM 1-43, To-Pro-3) allow rapid visual assessment of the regenerative process. Such studies have shown that regenerative hair cell progenitors arise from surrounding support cells which purportedly can repopulate lost hair cells through both proliferation-dependent and -independent mechanisms [8].

Genetic screens have succeeded in identifying regeneration-deficient mutants incapable of replacing hair cells [9]. Additionally, chemical modulators that enhance or inhibit this process were recently identified through large-scale compound screens [10], thus providing further molecular insights into which signaling pathways are involved in hair cell regeneration. Similarly, studies in which melanocytes were chemically ablated ultimately revealed that stem cells responsible for melanocyte regeneration are regulated via *kit* receptor tyrosine kinase signaling [11].

Other ‘cell toxins’ have been used to ablate different cell types (e.g., neuronal subtypes); however, the specificity of such reagents is often problematic. Thus, improved cell ablation techniques have been sought to expand cellular regeneration studies to nearly any desired cell type. Systems promoting the loss or functional compromise of distinct cell types, allow tests of cellular function (e.g., neural subcircuitry ‘dissection’), and facilitate investigations of mechanisms permitting cell-specific regeneration.

3 Cell-Specific Ablation Methodologies

Both chemically induced and light induced (e.g., laser ablation) cell ablation paradigms are applied in modern regenerative biology. Their relative strengths and weaknesses facilitate different questions and/or assay endpoints (summarized in Table 1). Typically, chemical ablation provides a less rapid approach with regard to cell death onset, and can also suffer from potential off-site effects (e.g., ill-defined cell ‘toxins’). However, the ability to elicit cell loss in large numbers of fish makes this approach applicable to large-scale genetic and/or compound screens. As the entire organism is typically exposed to chemical treatments, utilizing transgenic targeting methodologies in conjunction with chemical ablation can limit undesired “off-site” effects; however, this approach requires additional time to create appropriate toolsets. Light induced ablation techniques offer a rapid onset, but reproducibility also typically requires cell-specific targeting methods (e.g., transgenic reporters, dyes, etc.).

With regard to cell function assays, chemical ablation, by permitting the elimination of defined cells in a large number of fish simultaneously better facilitates studies requiring a large number of samples (e.g., behavioral tests). Conversely, light induced methods are ideal for single cell scales and for high-resolution imaging of cellular responses (e.g., effects on neighboring cells) due to the strict control over the timing of cell loss.

4 Cell-Specific Ablation Systems

4.1 Light/Laser Ablation

The zebrafish retina provides an ideal system for light ablation as 1), photoreceptor cells are sensitive to intense light and 2), optical transparency can be prolonged via chemical/mutant inhibition of pigment formation into larval and adult stages, respectively. Photoreceptors are especially vulnerable to damage due to their proximity to the retinal pigment epithelium which absorbs the majority of light energy. Thus, prolonged intense light treatments can be used to destroy photoreceptors but spare the inner retina from damage [4].

An alternative method involves Argon laser photoablation. Again, due to their relative sensitivity to light this technique allows selective ablation of photoreceptors [12]. This approach illuminated a role for inner nuclear layer progenitor cells during retinal regeneration. Photoreceptor losses lead to lesion-localized proliferation of microglia, retinal progenitors, and Müller glia – the latter cell type migrated from the inner to the outer nuclear layer, implicating them in the regeneration of cone photoreceptors. Combining reductions in laser intensity and/or exposure times with fluorescent reporter based cell targeting, it is possible to induce highly localized cell loss [13]. This approach can be coupled with confocal imaging to assess immediate effects of cell loss on neighboring cell types and/or resident stem cell niches.

A more widely applicable approach allows ablation of any fluorescently labeled cell type by illuminating fish with the appropriate excitation wavelengths, thereby achieving cell specific ablation of the labeled cells [14,15]. Photoactivatable cell toxins (e.g., KillerRed) provide another example of this methodology [16]. The power output of the laser and the optical

precision of the system determine the overall accuracy of ablation, i.e., the amount of bystander death. This must be carefully calibrated and may require an investment in pulse-laser and/or multiphoton laser technology for precise, single-cell ablation [15].

4.2 Chemical Ablation

Chemical ablation models have been applied to a broad range of regenerative studies. For instance, cellular toxins have been used in an effort to illicit cell-specific losses (e.g., ablation of melanocytes, olfactory epithelium, and pancreatic -cells). If such toxins function through unique or well-defined mechanisms the possibility of cell-specific death arises. For instance, using 4-(4-morpholinobutylthio)phenol (MoTP) to ablate melanocytes relies on tyrosinase activity within melanocytes/melanoblasts to convert MoTP to a cytotoxic quinone. Other toxins work through a disparate array of signalling pathways making it important to fully account for the possibility of non-specific cell loss with each. Indeed, some toxins initially assumed to be highly selective have been shown to non-specific or to elicit so-called “bystander effects” – the death of cells surrounding the targeted cell type. Still, when properly targeted, toxin-based studies have succeeded in providing important insight into how specific cell types are replaced.

There are numerous chemical ablation paradigms that are applicable to the zebrafish system. Each approach has distinct strengths and weaknesses due to mechanistic or procedural diversity (summarized in Table 2). The following sections detail some of the cell-specific ablation paradigms that have been developed to date.

4.2.1 MoTP – Melanocyte Ablation—The Johnson laboratory identified MoTP as a compound capable of selectively ablating melanocytes [11]. Using this reagent, they demonstrated that, post ablation, a variable number of melanocyte precursors (35-145) give rise to approximately 350-400 melanocytes upon completion of regeneration [11]. MoTP also facilitated a forward genetic screen that identified two mutants with stage-specific defects in melanocyte regeneration. Importantly, this screen was the first concrete evidence that the pathways necessary for cell genesis are – at least to some degree – separable from those required for cellular regeneration. Surprisingly, this separation extends to factors regulating the proliferation of melanocyte progenitors; *skiv2l2* (superkiller viralicidic activity 2-like 2), a predicted DEAD-box RNA helicase, is required for melanoblast proliferation during regeneration but not during embryogenesis [17].

4.2.2 Triton X-100 – Olfactory Epithelium Ablation—Triton X-100 detergent has been utilized to study neuronal turnover in the olfactory epithelium (OE) of mammals and, more recently, zebrafish [18]. Like hair cells, the OE is particularly susceptible to damage from soluble reagents due to its exposed nature. Triton X-100 (0.7%) was injected unilaterally into the right olfactory naris of adult zebrafish, allowing the contralateral side to serve as an internal control. A two minute application resulted in a significant loss of all three sensory neuron subtypes of the OE (ciliated, microvillous, and crypt) and loss of sustentacular cells – presumably by disrupting cell membranes as detergent treatments are typically used to solubilize membrane proteins. All cell populations that were significantly reduced post injury recovered fully by 5 days post-treatment. This technique has the advantage of being effective following very short treatment durations. However, in zebrafish, specificity may require localized application (e.g., injection) as other surface cell types (e.g., neuromasts and possibly the enveloping layer of the epidermis) would likely be effected by upon full immersion.

4.2.3 Streptozotocin – Pancreatic beta cell Ablation—Streptozotocin is an alkylating agent similar in structure to glucose, and is a potent reagent with regard to the

selective destruction of pancreatic β -cells (via GLUT2 specific transport). Intraperitoneal injection of streptozotocin has been used extensively in rodents to create Type I Diabetes models [19,20]. Ideally, immersion in streptozotocin could be used to create similar models in fish model systems. Unfortunately, data suggest that such treatment regimens are ineffective in fish [21]. Nevertheless, injection of a single high dose of streptozotocin does serve to eliminate pancreatic β -cells in adult zebrafish [22]. Post injection, glycemic levels rose significantly but were restored to normal within 2 weeks, corresponding to islet regeneration and repopulation of β -cells from Pdx1-positive precursors. Although streptozotocin is effective upon injection, and thus useful for characterization of the regenerative process, such treatment regimens are incompatible with large-scale genetic or chemical screens aimed at defining factors that are essential for and/or promote β -cell regeneration.

4.2.4 Aminoglycoside/Antibiotic – Hair Cell Ablation and Kidney Injury—

Aminoglycosides like neomycin function by generating free radicals in hair cells; they can be vestibulotoxic (e.g., dizziness, nystagmus) or cochleotoxic (hearing loss) in humans [23]. Hair cells of the lateral line in zebrafish are acutely sensitive to ototoxic compounds like neomycin, providing a cost-effective yet highly selective approach for studying hair cell regeneration. Application of 0.5mM neomycin for 4 hours has been shown to eliminate DASPEI labeled hair cells in neuromasts [7]. Additionally, neomycin treatment has been utilized for genetic and small molecule screens aimed at delineating key molecular components and discovering potential regenerative therapeutic compounds, respectively [10,24].

Another aminoglycoside, gentamycin has been utilized to study acute kidney injury (AKI) in zebrafish. The zebrafish pronephric kidney consists of nephrons that are conserved in higher vertebrate species, including mammals. Gentamycin ablation of kidney epithelial cells in zebrafish larvae is often lethal – which presents a barrier to temporal analysis of epithelial regeneration in this organ. Consequently, recent studies have transitioned to a more region specific laser ablation methodology (as discussed prior). This technique provides greater control of spatial and temporal parameters of kidney injury, and is amenable to different cell types/structures within the kidney [25].

4.2.5 Dexamethasone – T-cells—Dexamethasone is a synthetic glucocorticoid agonist, and is a vastly more potent immunosuppressant than cortisol or prednisone. In larval zebrafish, dexamethasone treatment has been demonstrated to effectively ablate T-cells. This compound was utilized to delineate the spatial and temporal aspects of T-cell ablation and repopulation/homing to the thymus following transplantation [26].

4.3 Transgenically Targeted Cell Ablation Systems

Several systems have been developed that couple transgene expression to cell ablation. Such methods come in two general forms, those expressing genes that elicit cell death directly (e.g., diphtheria toxin, caspase) and those expressing enzymes which convert otherwise innocuous substrates into cytotoxins (e.g., thymidine kinase, nitroreductase). The former require inducible transgene expression (or inducible protein activation) strategies to allow temporal control over the induction of cell death, while the latter control the timing of ablation by requiring prodrug substrate applications. These systems offer several potential advantages: 1) Specificity – can be targeted to nearly any cell type of interest, 2) Inducibility – those versions involving substrate dependent ablation, 3) High-volume – ablation can be induced simultaneously in thousands of fish – a critical element for applying HTS methods and, 4) Ease of use – once appropriate lines are derived ablation is induced simply by adding reagents to water. In the sections below we detail these methodologies and discuss inherent

strengths and weaknesses that shape how each system can be most effectively applied (summarized in Table 2).

4.3.1 Diphtheria Toxin—Transgenic expression of diphtheria toxin (bacteriophage origin, *Corynebacterium diphtheriae*, encoded by the *tox* gene) has been successfully applied to the analysis of pancreas development in the absence of exocrine tissue [27]. A transgenic zebrafish line was established with a construct in which diphtheria toxin expression is under control of the *elastaseA* promoter, targeting the toxin to exocrine pancreas. In these lines exocrine tissue is constitutively ablated, while leaving neighboring endodermal cells unaffected. As in this case, effective use of diphtheria toxin requires a promoter that is exclusive to the targeted cell type – i.e., completely silent in other cells. A variation of this approach allowing temporal control of toxin expression (via the inducible Cre-lox system) was later utilized to study cardiomyocyte regeneration in zebrafish [28]. Injection of, or incubation with 4-hydroxy-tamoxifen was utilized to activate cardiomyocyte-specific expression of diphtheria toxin resulting in dose-dependent death of heart tissue. With this technique, a significant delay in the onset of cell death was observed (2 days post injection). This is likely a result of the mechanism of action of diphtheria toxin, essentially acting as an inhibitor of protein synthesis through inactivation of eukaryotic elongation factor-2 and thereby arresting RNA synthesis. One potential drawback of this system is the extreme potency of the toxin which, in the absence of strict control over expression levels, can cause off-site injuries.

4.3.2 Caspase—Caspases act through classical programmed cell death pathways, thus caspase-based ablation systems provide ideal models for diseases involving apoptosis. Toward that end, inducible expression of initiator Caspase-8 was developed to create a novel cell specific ablation model [29]. A similar system facilitating inducible activation rather than expression was created by fusing initiator Caspase-8 (or 9) with the mutant estrogen receptor (Caspase-8-ER^{T2}) [30]. This system has several advantages including extremely rapid onset of cell death; in cell culture assays elevated Caspase-3/7 activation was evident 1 hr after tamoxifen exposure with cells exhibiting signs of apoptosis by 3 hrs [30]. Although this system was shown to be operable in human, mouse, and zebrafish cell cultures, it has not yet been shown to work *in vivo* in the zebrafish system.

4.3.3 Thymidine Kinase/Ganciclovir—Transgenically expressed thymidine kinase (derived from the herpes simplex virus) renders targeted cells susceptible to ganciclovir-induced cell death. Thymidine kinase phosphorylates ganciclovir which then inhibits DNA polymerase activity, arresting the cell cycle and resulting in the death of dividing cells. This is a clear limitation of this method for regenerative research; it is only effective for eliminating proliferating cell populations. However, therapeutically, this has advantages within the realm of chemotherapy [31]. Similarly, this ‘limitation’ could be leveraged to test the plasticity of regenerative responses regarding the relative roles of proliferative and non-proliferative (i.e., transdifferentiation) mechanisms of cell replacement. However to date, like the caspase system, this methodology has not been adapted to the zebrafish system.

4.3.4 Nitroreductase—We and others have adapted the nitroreductase (NTR; bacterial origin, *Escherichia coli*, encoded by *nfsB* [32]) based system of genetically targeted cell ablation [33] to the zebrafish system [34,35,36]. NTR converts innocuous prodrug substrates, such as metronidazole (MTZ), into cytotoxic DNA crosslinking agents – providing cell-specific ablation of the targeted cell type [37].

Exemplifying the versatile nature of transgenically targeted ablation strategies, the NTR system has been used to selectively deplete multiple cell types in zebrafish, including: pineal

photoreceptors [38], pancreatic beta cells [21,34], cardiomyocytes [34], hepatocytes [34], macrophages [39,40], podocytes [41], gonadal cells [42,43,44], skin cells [45], regionally defined habenular neuron subtypes [46], and retinal neuron subtypes [47,48,49]. Together, such platforms begin to create a framework to compare molecular mechanisms regulating regeneration at the level of individual cell types. For instance, we have recently initiated studies to compare gene expression changes (microarrays, RNA seq, and qPCR) occurring during the loss and regeneration of two different NTR targeted retinal neuron subtypes, bipolar cells and rod photoreceptors.

Similar to “Caspase suicide gene” systems, the mechanism of cell death downstream of NTR/MTZ-induced DNA crosslinking appears to occur through classic apoptotic mechanisms; marked by TUNEL/activated Caspase-3, dependent on *tp53* expression and oxidative stress [45]. Also like caspase and diphtheria toxin, the NTR system is independent of the cell cycle allowing ablation of quiescent cell types such as neurons. One aspect that sets NTR apart from other transgenic targeting methods is relative cost, e.g., MTZ is a more cost-effective reagent than 4-hydroxy-tamoxifen. Another key advantage of the NTR system which our laboratory has sought to take full advantage of arises from the fact that fusion proteins linking NTR and reporter transgenes retain both enzymatic and fluorescence activity [50]. This allows regeneration kinetics to be easily followed using methods to detect relative levels of NTR-XFP fusions in living zebrafish over time. In turn, this facilitates both genetic and chemical screens. For instance, we are currently performing a small-scale forward genetic screen to identify regeneration-deficient mutants, larvae that fail to replace NTR-YFP expressing photoreceptors. Intriguingly, on the drug screening side, visual assessment of the relative numbers of NTR-XFP expressing cells following MTZ-induced ablation was recently used to identify compounds that promote the regeneration of pancreatic beta cells [51].

Ultimately, methods facilitating automated quantification of *in vivo* fluorescence levels would allow regeneration-based screens to reach new levels of throughput. Accordingly, we developed an HTS system for rapidly quantifying regeneration kinetics, termed ARQiv (automated reporter quantification *in vivo*). ARQiv provides a rapid (> 50,000 units/day), accurate, and reproducible (Z factors of > 0.5) method employing standard HTS instrumentation (fluorescence plate readers) to automate detection of fluorescent reporters in living zebrafish [52]. Combined, the NTR and ARQiv systems facilitate discovery of compounds which promote regeneration; i.e., potential therapeutics. In addition, NTR-XFP fusions facilitate high-resolution time-lapse visualization [47] of the regenerative process: the kinetics of targeted cell loss/replacement and interactions with neighboring cell types (e.g., stem cells). Thus, NTR-XFP fusions provide perhaps the most complete view of the cellular mechanisms of cellular regeneration available in a vertebrate species.

5 Method: Long-term Time-lapse Imaging of Regenerative Processes in Larval Zebrafish

5.1 Overview

Our laboratory performs the majority of our regenerative studies in larval zebrafish (e.g., 6-14 days post fertilization, dpf). The primary advantage of this approach is that the entire regenerative process can be monitored directly, allowing integration of multiple perspectives – e.g., stem cell activation/proliferation, progenitor cell differentiation, and regenerative outcomes. Such prospective assay platforms improve quantification by circumventing inherent variability between individuals, i.e., variation in the number of cells labeled and/or relative brightness. Observing regeneration in real time also facilitates a number of studies that are otherwise difficult to address. For instance, transdifferentiation – the direct

conversion of one cell type to another – has recently been implicated in multiple regenerative paradigms. However, transdifferentiation is extremely difficult to document in the absence of direct observation. Similarly, investigating roles played by neighboring or invasive cell types is facilitated by rapid time-lapse imaging. Accordingly, we have developed methods for *in vivo* high-resolution time-lapse imaging that span temporal resolutions ranging from minutes to days [47,53]. Here, we will detail recent improvements we have made which extend the total amount of time that rapid time-lapse studies can be performed and thus provide improved “windows” into regenerative processes in larval zebrafish.

5.2 Creation of NTR-XFP and Other Complementary Transgenic Lines

The first step in preparing time-lapse based regeneration studies is to determine if appropriate resources are available to: 1) label and, 2) kill the cell type(s) of interest. While some cell types can be labeled and killed specifically with soluble reagents (e.g., aminoglycosides and hair cells) most require transgenic targeting. The NTR-based transgenic system is highly amenable to time-lapse imaging as it couples both requirements via co-expression of NTR and reporters. This strategy – targeting individual cell types with NTR-XFP fusion proteins – is applied in our laboratory to visualize the ablation and subsequent regeneration of retinal bipolar [47] and rod photoreceptor cells [52].

5.3 Exclusive Targeting of Specific Cell Types

It is important to note that the criterion for what constitutes an appropriate promoter for establishing transgenic lines for cell-specific ablation is more stringent than for cell labeling. In order to ensure that cell loss is limited to the intended target it is necessary to identify promoter/enhancers that are *exclusively* active in the desired cell type. Unfortunately, many published “cell-type specific” transgenes are typically discussed only within the context of a given tissue or region – expression in other tissues is often the case. Bacterial artificial chromosomes (BACs) offer improved cellular specificity and can be used to produce stable transgenic animal models, albeit at low efficiency and potentially integrating as multi-copy concatamers. Modification of this technique through application of high capacity *Tol2*-mediated transgenesis has succeeded in integrating single copy of BAC transgenes which display excellent expression specificity [54], however BAC transgenesis efficiency tends to remain significantly lower than with smaller plasmids. However, as noted above, the number of defined promoters that provide cell exclusive targeting is limited. To address this issue, we have begun to explore the use of “silencers”, regulatory elements which turn off transgene expression in all but a select tissue type. Our findings using the neuron restrictive silencer element (NRSE) [55], suggest that creating novel associations between regulatory activators (e.g., enhancers) and silencers will provide a means to attain cell-type exclusive transgene expression for cases that might otherwise prove difficult to target..

5.4 Bipartite Transgenic Systems Increase Expression and Versatility

Another issue that can compromise the usefulness of zebrafish transgenic lines is weak expression. The Gal4/UAS system was adapted to zebrafish, in part, to overcome this issue [56,57]. This ‘bipartite’ system allows combinatorial libraries of transgenic resources to be established by separating transgene expression into two complementary components: 1) The “driver” element – controlling where and when transgenes arise (e.g., Gal4 transcriptional activators) and, 2) The “reporter/effector” element – determining what and how much transgene product is produced [e.g., UAS (upstream activating sequence), binding sites for Gal4, based lines]. Crossing a given driver line with different reporter/effector lines allows a versatile array of experiments to be performed in driver targeted tissue/cells. Similarly a single reporter/effector line may be crossed to a variety of driver lines, allowing the same assay (e.g., ablation) to be applied to an array of different cell types. Thus, a 14xUAS:NTR-

mCherry effector line was established to facilitate regenerative studies in a variety of cell/tissue types [58]. However, this line is susceptible to trans-generational silencing (e.g., reduced and/or mosaic expression) which has been correlated with methylation of repeated UAS sequences [59]. New UAS:NTR-XFP lines either reducing the number of UAS sites [60] or incorporating non-repetitive UAS sites [59] could be derived to circumvent this issue. Finally, although extremely powerful in theory, a general lack of expression specificity has been observed in many Gal4-based zebrafish driver lines. As noted above, this hinders manipulative applications such as cell ablation. Importantly, the “silencer” strategy discussed above also promotes improved expression specificity within the context of Gal4-based enhancer traps. In the case of NRSE, this effectively limits trapped expression patterns to neuronal tissues through a mechanism that is dependent on expression of the NRSE binding factor, REST [55].

5.5 Pigmentation Mutants Facilitate Late Larval Imaging

Depending on the target tissue, utilizing transgenic lines in ‘transparent’ mutant backgrounds may be advantageous for imaging and/or quantification. For example, *in vivo* confocal imaging of the zebrafish retina at late larval stages is hindered by both iridophores and melanophores. Thus, we typically derive transgenic lines in pigment mutant backgrounds to ameliorate this issue, e.g., *roy orbison* (*roy*) mutants reduce iridophore numbers without overtly impacting visual performance. However, for late larval stage applications it becomes necessary to eliminate melanophores as well – as prolonged PTU exposure can have deleterious effects. Double mutants (*roy;albino*) can be used for this purpose although as with any mutant, potential differences from wild type must be accounted for. In the case of *roy;albino* mutants, deficits in visual performance have been observed [61].

5.6 Imaging and Targeting Interacting Cell Types

Time-lapse imaging can be extremely insightful for characterizing the “behavior” of individual cell types (e.g., neuritic outgrowth patterns [53]). However, to fully account for more complex biological processes, such as regeneration, methods that allow multiple interacting cell types to be monitored simultaneously and/or manipulated independently are required. Accordingly, we adapted multi-color transgenic labeling techniques to monitor cell-specific loss and regeneration in tandem with retinal stem cell subtypes [62]. Other cell types likely play significant roles in regeneration as well. We are particularly interested in studying how the response of innate immune cells to retinal cell loss impacts regeneration.

A recent study employed the NTR system to explore roles played by macrophages and neutrophils in a fin regeneration [40]. Interestingly, ablation of macrophages (but not neutrophils) impaired resolution of the inflammatory response and inhibited regeneration – conversely, ablation of neutrophils (but not macrophages) accelerated regrowth of the injured fin [40]. These examples exemplify the variable roles that the innate immune system can play in regenerative paradigms.

We obtained transgenic lines that allow similar studies in our system (kind gift of Dr. Chao-Tsung Yang, Ramakrishnan Laboratory, University of Washington, Seattle). These lines utilize a *mpeg1* (*macrophage expressed gene 1*) promoter that facilitates improved targeting of macrophages [63]. Combined with NTR ablation of retinal cell subtypes, these resources have availed preliminary investigations into how resident retinal macrophages interact with apoptotic photoreceptors (Figure 1). Additionally, we seek to resolve the timing of NTR-based cell death following MTZ exposure by assessing the time at which macrophages first begin phagocytosing cell debris (Figure 1). Such experiments necessitated the need to monitor immune cell/photoreceptor interactions at high temporal resolutions over long

periods of time. Initial imaging protocols [53] proved inadequate for monitoring highly migratory cells (e.g., macrophages) on minute to minute time scales due to viability issues. To overcome this limitation, a temperature controlled media ‘flow-through’ system was established that permits imaging over extended durations, i.e. 2-3 days continuously.

5.7 Establishing an Effective Ablation Protocol

Once appropriate NTR related transgenic resources are in hand, a number of issues can arise including: 1) Non-specific Death – although MTZ is known to promote targeted cell death, we have noted that injuries involving the loss of large numbers of NTR-expressing cells can produce “bystander” effects after the initial wave of cell loss i.e., subsequent death of nearby cells, 2) Toxicity – although 10mM MTZ solutions are routinely employed in zebrafish, this concentration is close to levels causing deleterious effects in larva (e.g., edema) and, 3) Perdurance – NTR-XFP fusions can take considerable time to decay, moreover, fragmented cells can appear intact at resolutions below those facilitated by confocal microscopy. Thus, early in the development of any new NTR/MTZ-based ablation paradigm a series of control experiments, outlined below, are necessary to establish parameters that promote robust and reproducible cell ablation while avoiding possible complications.

5.8 Verifying Cell-specific Death

Because MTZ conversion initiates classic apoptotic mechanisms [45] standard reagents can be used to mark dying cells (e.g., TUNEL, activated-Caspase 3 immunoreactivity, Annexin V-YFP transgenic lines [64]) and co-labeling with NTR-linked reporters can thus validate targeting specificity. Reporter perdurance actually aids this effort. In our experience, evaluating cell death immediately after a 24 hr MTZ exposure provides ample overlap between cell death markers and fragmented/decaying NTR-XFP reporters. To eliminate the possibility of non-specific death, evaluations at 48 and 72 hrs post-MTZ are also required to account for possible lags in “bystander” effects.

5.9 Determining Optimal MTZ Treatment Regimens

Because 10mM MTZ treatments can have deleterious effects on the general health of larval fish, we recommend evaluating whether lower concentrations and/or reduced exposure times are effective on targeted cell types. In particular, we have noted a positive correlation between the relative metabolic activity of the targeted cell and MTZ effectiveness (e.g., highly active photoreceptor cells can be ablated with 2.5mM MTZ, while relatively quiescent cells such as lens and notochord are more difficult to eliminate). In addition, because prodrug substrates are consumed during treatment, the volume of MTZ solution should be adjusted for the total number of fish treated (we have found ~1 ml per larva to be sufficient, e.g., 25mL MTZ solution per 25 embryos incubated in a 100mm × 20mm plate). Characterization of the time course of ablation can help to maximize animal viability and reduce excess stress by determining whether reduced MTZ concentrations/exposure times are effective. Methods for marking dying cells (as above) can be used at intervals beginning as early as 4 hrs post-MTZ application. However, this only indicates whether cell death has been initiated. To determine whether reduced MTZ treatment regimens are sufficient for ablation it is also necessary to verify that appropriate numbers of the targeted cell types are lost using methods for quantifying cell numbers (e.g., confocal microscopy) and/or fluorescence levels (e.g., ARQiv method – 1 fish per well in a 96-well plate, Greiner Bio-one, 651209).

5.9.1 Preparing Reagents—MTZ is not readily soluble at 10mM concentrations. DMSO is typically used to promote solubility, however, we prefer to avoid possible deleterious effects this reagent can have, including induction of stress responses (e.g., *hsp70* activation)

at concentrations as low as 0.1% [65]. MTZ has the distinct benefit of being bioavailable without the addition of DMSO. However, MTZ solutions should be mixed for a minimum of 1 hour in the absence of DMSO (e.g., end over end rotation at 300 rpm) to ensure MTZ is fully solubilized. For larval applications we recommend diluting MTZ in a 0.3x Danieau's solution (Table 3). If 10mM MTZ treatments fail to induce ablation of a given cellular target, adding 0.1% DMSO can be used to increase larval permeability.

6.1 Long-term Time-lapse Imaging of Larval Zebrafish During Cell Loss and Regeneration adapted from Ariga et al. 2010 [62]

Several hours prior to imaging, dissolve low melt agarose (LMA; Fisher®, BP1360-100) in embryo medium (e.g., 0.3x Danieau's solution) to a final concentration of 0.5% (this concentration does not impede MTZ diffusion – a range of 0.25-0.5% LMA provides similar viability) and store at 40 °C. At least 30 minutes prior to imaging, prepare a mounting solution: 0.5% LMA + anesthetic (e.g., tricaine methanesulfonate, 756µM final, Fisher®, NC9673832) + 1-Phenyl-2-thiourea (PTU, administered at 16 hpf for imaging the eye, 200µM final, TCI America®, P0237 – if required to inhibit melanophore formation) – mix and return to 40°C. Anesthetize fish by transferring them into embryo medium containing anesthetic/PTU and allow time for the fish to become non-responsive to touch (~3min). Transfer individual fish to mounting medium and then, in a 25-30µL drop of mounting medium, to a 100mm × 20mm plate (e.g., Corning® Treated Cell Culture Dish, CNG-430293) for imaging. After agarose has solidified, transfer the plate to the confocal stage (upright microscope) and add embryo medium containing: anesthetic, PTU (if required), MTZ (at predetermined concentration, Fisher®, AC2034-1000), and antibiotics (e.g., Penicillin 5 IU/mL-Streptomycin 5µg/mL final, MP Biomedicals®, 1670049) for extended imaging times (>16 hours) to prevent bacterial growth. If desired, MTZ incubation can begin at an earlier timepoint but should then be included in all mounting solutions to provide persistent exposure.

6.2 Live-imaging for extended periods of time in late-stage larval zebrafish

Applying the mounting methodology as above, the addition of a stage warmer provides a consistent temperature (e.g., 28.5°C). We developed an alternative cost-effective 'flow-through' method that has the added benefit of increasing the amount of time LMA mounted larvae remain viable. Our approach uses a pump to circulate warmed media through the sample plate. A peristaltic pump is ideal for this as it limits pulsatile movements which can lower image quality. Economic options are available (e.g., Fisher Scientific® Variable-Flow Chemical Transfer Pump, 355usd). Also required: 1x 500-600mL beaker, thermometer, water bath (15cm depth with at least 2L capacity), 5m of polyethylene tubing (e.g., Intramedic® Non-Radiopaque PE tubing, I.D. 2.69mm O.D. 3.50mm – fits securely with the pump above), and adhesive to secure tubing to bottom of plate (e.g., Darice® adhesive foam sheets). Figure 2 provides a schematic of the temperature controlled 'flow-through' system.

Obtaining a stable 28.5°C temperature in the imaging plate requires initial calibration of the system due to the following variables: pump flow rate, water bath temperature, and ambient room temperature. Modulating these variables in sequence (i.e., finding the appropriate flow rate prior to adjusting water bath temperature) permits timely calibration. Ideally, calibration should be completed at least one day prior to the extended-period imaging. Once these variables have been resolved, barring any significant change in room temperature, subsequent experiments will provide equivalent conditions. The PE tubing and pump should be primed (i.e., void of air bubbles) prior to image capture.

There are several advantages to this system: 1) maintenance of ideal working temperature of 28.5°C, 2) improved oxygen exchange through the liquid-gas and liquid-agarose interface

via constant movement of media, 3) the total media volume can be effectively increased 2-3 fold over normal recovery conditions, thereby diluting any phototoxic byproducts (e.g., reactive oxygen species). In our experience, this simple set up allows continuous imaging of mounted larvae for 2-3 days, in turn facilitating investigation of a host of questions regarding the relative roles played by different cell types in the regenerative process.

Conclusion/Summary

The advancement of regenerative biology is intimately linked to the development of novel tools that can evaluate the regenerative process in its entirety. Cell-specific regenerative paradigms can facilitate such studies by focusing on individual stem cell niches. A number of chemically induced ablation methodologies exist that facilitate investigations of a diverse array of injury models and degenerative disease states. We contend that such cell-specific ablation models can provide unique insights into how regenerative systems respond to and repair the loss of individual cell types.

The NTR system of prodrug inducible cell ablation provides a versatile, cost-effective means of ablating genetically targeted cell types in zebrafish that is free of limitations regarding cell type or cell-cycle dependence, and is scalable to HTS applications. Comparisons were drawn to other ablation methods, highlighting the amenability of the NTR system to integrate with powerful high-resolution time lapse imaging (Figure 1) and HTS screening technologies [52]. With continued development of the NTR and similar systems in zebrafish – a quintessential organism for current regenerative biology studies – a great deal can be learned regarding molecular mechanisms regulating regenerative processes, in turn providing potential inroads to curative therapeutics for degenerative disease.

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Highlights

- Cell-specific ablation provides new models for regenerative research in zebrafish
- Nitroreductase based ablation facilitates large-scale genetic and chemical screens
- Time-lapse imaging can provide unique insights into regenerative processes
- Cost-effective 'flow-through' system extends viability during time-lapse
- Innate immune system responds to circumscribed loss of specific cell types

Figure 1. Kinetics of rod photoreceptor ablation and macrophage activation via MTZ

Confocal time-lapse microscopy (Olympus FV1000) was used to characterize cell loss and neighboring cell responsiveness parameters following MTZ-induced ablation of rod photoreceptor cells in the retina. Two transgenic lines were crossed to label photoreceptors (PR) with a NTR-YFP fusion protein [yellow, *Tg(rho:YFP-NTR)gmc700*], and macrophages (MP) with RFP [red, *Tg(mpeg1:RFP)*] [63]. A 6 dpf double transgenic zebrafish was imaged immediately after MTZ treatment. Temporal resolution: a z-stack encompassing the majority of the imaged retina and surrounding tissue was collected every 10 minutes (5 min laser, 5 min 'dark'). Ablation onset first became evident at ~300min after MTZ exposure – interpreted as separation of outer and inner segments of PR cells. **A-B:** Arrows indicate MP cells near the eye that transitioned from resting (ramified) state to migratory (activated) state over the course of the study. **C-F:** (1.5x zoom of ROI, yellow box above): Green/Cyan traces illustrate the migration path of two MP cells within the eye, image acquisition times corresponding to migratory traces are indicated at the lower right. MP#1 was stationary for 630min (C) and migrated from 640min-920min (D-F); MP#2 was stationary for 410min (C) and migrated from 420min-920min – in each case, the onset of migration coincided with the death of nearby PR cells. **D-F:** Arrowheads indicate an observed phagocytic event (D-E), and subsequent absence of the inner segment of a dead PR (E-F). Scale bar(s), 250μM.

Figure 2. Schematic representing the 'flow-through' system

The peristaltic pump (grey) provides consistent media flow. Media proceeds from the peristaltic pump to the water bath (blue) where heat transfer brings the media temperature up to $\sim 28.5^{\circ}\text{C}$. Water is circulated through the sample plate on the microscope stage. Media drawn from the sample plate proceeds once again to the peristaltic pump to repeat the cycle indefinitely. The increase in total media volume made possible with this system is believed to underlie temporal increases in specimen viability by diluting the accumulation of reactive oxygen species generated from prolonged periods of time-lapse imaging while maintaining a constant temperature.

Table 1
Comparison of Chemical and Light Induced Ablation Methodologies

Property	Chemically Induced Ablation	Light Induced Ablation
Temporal Dynamics	+ (slow or ill defined onset possible)	+++ (rapid)
Specificity	+ / +++ (off-site effects possible *)	+ / +++ (facilitated by labeling cell targets)
Sample Size	+++ (applicable to multiple fish simultaneously)	+ (labor intensive, limiting sample number)
Reproducibility	+++ (injury proceeds at a constant rate)	++ (variable injury possible)
Behavioral Studies	+++ (facilitated by larger sample sizes)	+ (limited by smaller sample sizes)
Imaging Studies	++ (dynamics can complicate assay design)	+++ (facilitated by improved dynamics)
Translational Impact	+++ (applicable to many degenerative diseases)	+++ (applicable to broad range of injury paradigms)

* typically problematic for toxins as opposed to transgenically targeted methods

Table 2
Comparison of Selected Chemical Ablation Methodologies

Methodology	CKRP Property	Range of Targets	Speed	Target Specificity	Scale/ Cost	Ease Of Use	Cell Cycle Independence
MoTP		+	+	+++	+++	+++	+++
Triton X-100		++	+++	++	++	+	+++
Streptozotocin		+	++	+++	++	+	+++
Aminoglycoside		+	+++	+++	+++	+++	+++
Dexamethasone		+	++	+++	+++	+++	++
Thymidine Kinase/Ganciclovir		++	++	+++	+++	+++	+
Diphtheria Toxin, 4-OHT		+++	+	++	++	+++	++
Caspase, 4-OHT		+++	+++	+++	++	+++	+++
Nitroreductase-MTZ		+++	++	+++	+++	+++	+++

Table 3
List of Materials for 6.1 and 6.2

Reagents:
0.3× Danieau's solution:
- sodium chloride, 58mM final (Fisher, S271-1)   - potassium chloride, 0.7mM final (Fisher, P217-500)   - magnesium sulfate, 0.4mM final (Fisher, M65-500)   - calcium nitrate, 0.6mM final (Fisher, C109500)   - HEPES, 5mM final (Fisher, BP310500)
Other reagents:
- Low melt agarose (Fisher, BP1360-100)   - Tricane methanesulfonate (Fisher, NC9673832)   - PTU (1-Phenyl-2-thiourea, TCI America, P0237)   - Metronidazole (Fisher, AC2034-1000)   - Penicillin-Streptomycin, 5 IU/mL-5µg/mL final (MP Biomedicals, 1670049)
2. Supplies:
- Dumont #5 fine forceps (Fisher, NC9404145)   100mm × 20mm plate, (Corning, CNG-430293)   - Polyethylene tubing (Intramedic, non-radiopaque PE tubing, I.D. 2.69mm O.D. 3.5mm, PE 260-7456)   - Beaker 600mL (Fisher, FB-100-600)   - Foam sheet white (2mm thick, Darice, 1144-13)
3. Equipment:
- Zoom stereoscope (Olympus, SZ51)   - Confocal microscope (Olympus, FV1000)   - Digital block heater – Isotemp (Fisher, 11-715-125DQ)   - Water bath – Isotemp (Fisher, 286741-150)   - Peristaltic pump (Fisher, 15-07767)   - Thermometer (Fisher, 14-997)