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Sundar Atre

The field of nanotechnology has been rapidly developing new materials. These materials have become incorporated into consumer products and the environment after only minimal assessment of their effects on human health or the environment. The toxicity of these new nanomaterials remains unknown due to the unpredictable nature of the core material and its surface functionalization interactions. To understand the toxicity of nanomaterials, the embryonic zebrafish model is nearly ideal because it is a vertebrate model, is small size, external development, ability to produce numerous eggs, and has a completely sequenced genome. Due to the genomic similarity between humans and zebrafish, mechanistic studies are translatable to likely human effects. The largest bottleneck to implementing high-throughput nanomaterial toxicity testing is the tedious manipulation of the embryonic zebrafish in a systematic manner to obtain reliable results. This rate-limiting step provided an opportunity to utilize automated robotics for the highly repetitive tasks, which reduced errors, increased efficiency and made high-throughput screening practical at OSU. An automation system utilizing traditional macro-automation systems coupled to a microfluidic manipulator was used to move zebrafish embryos for high throughput screening of nanomaterial toxicity. In addition to moving embryos, the automated system was able to perform complex manipulations such as moving the embryo to a pulled glass needle to perform microinjection of antisense RNA to block the function of a specific gene. This system achieved a success rate as high as 99.5% when properly setup and calibrated. Thus, an efficient and rapid screening platform for nanomaterial toxicity was successfully created to establish the first vertebrate-based, high-throughput automation core at OSU.

Automated Manipulation of Zebrafish Embryos for High-throughput Toxicology Screening of Nanomaterials.

by
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APPROVED:

Major Professor, representing Industrial Engineering

Head of the School of Mechanical, Industrial, Manufacturing Engineering

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

David Mandrell, Author

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I would like to thank my family for providing me with the opportunity, unyielding support and the occasional threat that has enabled me to reach this point. Without them I would not have made it to where I am now, or any of the places I may go from here. This thesis is dedicated to them.

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Chapter 1 – Introduction

Nanotechnology

Nanotechnology has only recently evolved into an applied field filled with extremely diverse techniques and applications. Even in its infancy, the potentials are clear; nanotechnology could change almost every aspect of human life. Great innovations have already been developed in a broad range of fields such as electronics, healthcare, cosmetics, and engineering [1-5]. Even with nanotechnology still in its infancy, the global demand for nano-containing products is expected to exceed 2.9 trillion dollars by 2014 [6]. The Environmental Working Group estimates that at least 2 million pounds of nano-titanium dioxide alone are consumed in the United States each year and are used in close to 10,000 over-the-counter products including medicine capsules, nutritional supplements, food icing and additives, skin creams, oils, and even tooth paste [7]. The applications of nanotechnology will only increase as new innovations occur, resulting in increased environmental and human exposure to nanoparticles.

Richard Feynman fundamentally started nanotechnology during an American Physical Society meeting at Caltech. On December 29, 1959 Feynman gave a lecture entitled *There's Plenty of Room at the Bottom* in which he proposed a powerful new form of chemistry through the direct manipulation of single atoms. Feynman believed that a set of precision tools could be used to build and operate a smaller set of precision tools, which in turn would build and operate smaller sets of tools [8]. Norio Taniguchi later defined the term "nanotechnology" in 1974 in his paper *On the Basic Concept of 'Nano-Technology*. Taniguchi outlined several, now common, semiconductor processes such as thin film deposition and ion beam milling, and other ultra precision machining techniques. It was these ideas, sowed by these forefathers of nanotechnology that drove most of the interest until the early 1980's

It was not until 1981 at an IBM lab in Germany that the ideas of nanotechnology started to become reality. The creation of the Scanning Tunneling Microscope (STM) earned its creators, Gerd Binning and Heinrich Rohrer, the Nobel Prize in Physics in 1986, and ushered in the era of nanotechnology [9]. This technology enabled individual

atoms to be resolved and manipulated [10]. The creation of the STM directly led to the development of fullerenes in 1985 and carbon nanotubes in 1991 [11, 12]. These discoveries opened scientists' and engineers' eyes to the possibilities of creating precisely engineered nanomaterials.

Today, nanomaterials, typically defined as less than 100 nanometers (nm) in their largest dimension, are the backbone of the nanotechnology revolution. These materials are quickly finding uses in everything from stat- of-the-art medical treatments [13] to stronger automobile running boards [6]. The key attribute of these extremely small particles is that they have high surface area to volume ratios. This allows scientists and engineers to utilize unique quantum mechanical and surface phenomena, which cause these particles to possess drastically different physicochemical properties than those of the same material at the macroscale. Nanomaterials ranging from fullerenes to ceramic and metal nanoparticles, to nanoporous and semiconducting nanomaterials are as numerous as their uses.

Some of the earliest developed nanomaterials were fullerenes. Fullerenes are molecules that are entirely composed of carbon and have a hollow core. There are many variations of fullerenes, such as carbon nanotubes, and several forms of buckyballs illustrated in Figure 1 [14]. Fullerenes have been a major focus of extensive research and development due to their potential for medical, electronic, and chemical applications. Potential uses include binding antibiotics to specific bacteria and targeting specific cancer cells as well as use as a light-activated antimicrobial [15].

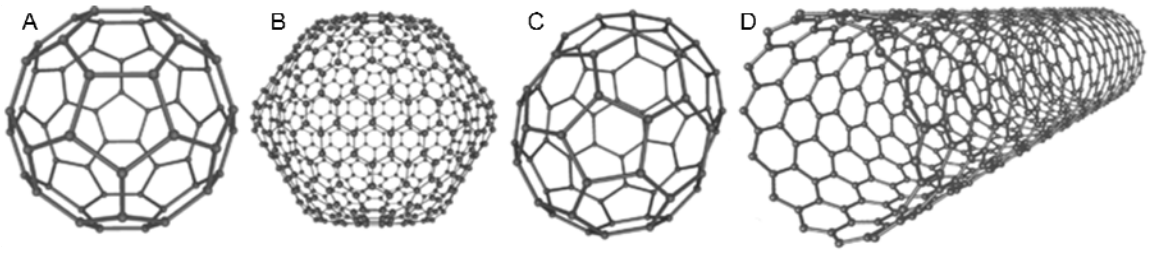


Figure 1 - Common fullerenes.
a) C60 buckyball b) C540 buckyball c) C70 buckyball d) Carbon nanotube. *Images adapted from Michael Ströck 2006*

Ceramic nanoparticles are one of the largest commercial uses of nanomaterials today. The market for ceramic nanoparticles is expected to be 1.5 billion dollars in

2010[6]. They are used in the slurry materials for structural composites and coatings to provide greatly improved strength and reduce the working temperatures required for sintering. Ceramic nanoparticles are also used for their unique thermoelectric properties which include metallically conductive composites[16].

Metal nanoparticles have proven useful in broad fields ranging from medicine to electronics. The high surface-to-volume ratio makes metallic nanoparticles valuable for catalysis processes utilizing rare and expensive materials; the physical properties of the nanoparticles allow more to be done with less material. The physico-chemical properties of the metal are exploited to engineer more precise nanoparticles. An application of this is antimicrobial nanosilver where nanoized silver is imbedded into products as an antimicrobial agent [17].

Nanoporous materials generally include particles bigger than 100nm up to bulk materials that have pore sizes on the nanometer scale. Nonporous materials are used as high performance insulators, optics, catalysis structures, separation media, and coatings. Their porous nature gives them an extremely high surface area compared to their volume. By functionalizing this surface, diverse catalytics and catalytic supports have been created.

Quantum dots, another nanomaterial, have become popular due to their unique properties. They have applications in transistors, solar cells, LED's, lasers, biotech imaging and more. Electronics applications take advantage of the Coulomb blockade effect of quantum dots to operate like a single-electron transistor. In computing, quantum dots are being developed for solid state quantum computation and combinations of quantum dots are being used to produce qubits as a new way of performing operations and quantum calculations. The largest commercial use of quantum dots are in fluorescence microscopy as they are 20-100 times more stable than other fluorescing materials [18].

Nanotechnology will continue to find new applications as new properties are discovered, new materials are generated, and other innovations occur. These innovations will enable the use of nanomaterials in more fields and applications. As these materials start to add benefits to an ever increasing number of facets of human technology and life, their use will become inevitably ubiquitous. This common use of

nanomaterials in everyday applications will cause an increased environmental and human exposure with largely unknown risks. [19].

Risks of Nanomaterials

As a vast array of new nanomaterials are being developed and marketed, addressing the risk of environmental and human exposure associated with classes of these new materials is paramount [20, 21]. Following the predictable but unfortunate juxtaposition of safety and commerce, new nanomaterials have transitioned from the laboratory to consumer products first, with questions regarding their toxicity surfacing second [22, 23]. With the incorporation of nanomaterials into most categories of consumer products, the public health risk, largely unknown, is well behind the development curve of new nano-chemistries. Growing public concern over nanoparticle safety mandates careful exploration of the possible biological consequences of human and environmental exposure to nanomaterials. The research into the toxicity of nanomaterials to date indicates that nanomaterials have safety issues. Assumed to be relatively safe, titanium dioxide (TiO_2) nanoparticles are used for implants in humans due to their biocompatibility and stability, and is used in medicine capsules, nutritional supplements, food additives, skin creams and oils, and toothpaste. But TiO_2nanoparticles have recently been reported to cause DNA damage and chromosomal breaks in laboratory mice 5 days after ingestion with water [7]. This is only one of many examples where nanomaterials with questionable safety are being incorporated into consumer products with little or no testing or government oversight.

With such a broad range of nanomaterials being manufactured, and with countless more in development, industry and academia need a testing regimen that can quickly address the toxicity of nanoparticles. If the immediate toxicological risk cannot be quickly determined, the perceptual risks, accurate or not, will drive regulations and public opinion of nanotechnology. The emerging field of determining the unique and complicated physicochemical effects of engineered nanomaterials on biological systems is called nanotoxicology.

Nanotoxicology

Understanding nanotoxicology will better enable major advances in safe and durable nanoparticle technology. The large challenge now facing the field of

nanotoxicology is to identify and categorize key characteristics that allow researchers to better predict toxicity of nanoparticles. At present, researchers have to examine each new material individually to determine risks that it may pose to humans and the environment. As more information is learned about the nanomaterial-biological interactions, researchers will be able to identify nanomaterials that pose a higher level of risk and target those for further screening. With further investigation, key structures that are indicative of adverse biological response can be identified, which will be informative to designing lower or no risk nanomaterials.

Determining the nanotoxicology of these new materials is of major importance. The potential for nanomaterials is so large that some have predicted that they will enable the third industrial revolution [24]. With extreme pressure from industry to quickly discover and push to market new NP based products, great care must be taken to prevent a repeat of one of the largest engineering mistakes of the past century, asbestos. Asbestos was lauded as a great new material and was adopted in a vast array of commercial uses and consumer products prior to its toxic effects being fully realized, leading to the mass dispersal and use of a very harmful material. An example of how some nanotechnology may fall into the same trap is carbon nanotubes. Carbon nanotubes are one of the flagship nanomaterials and early adopters are already producing consumer products containing them. The structure of carbon nanotubes is characteristically very similar to asbestos, and preliminary studies have indicated that carbon nanotubes pose many of the same risks as asbestos [13, 25]. Nanotechnology cannot risk following in the footsteps of the asbestos industry's mistakes. This will result in a negative public perception of nanotechnology. Stringent regulations would then likely overcorrect by stifling new nanotechnology development before the potential risk has been assessed and understood. To both promote safety and advance nanotechnology to meet pressing needs the research community, industry, and regulators need to work together to establish rapid, standardized toxicity assessments of the nanomaterials already on the market. This nanotoxicity screening needs to be cost effective and informative, while allowing for rapid high throughput assessment in order to have industry and regulators participate. It is up to those in the field of nanotechnology to safeguard the environment and public of their nanomaterials, and preserve the image and reputation of the industry that uses them.

Once the nanotechnology industry has implemented a rapid screening program, a method of sharing and correlating data from across the nanotechnology community will need to be incorporated. This collaboration in testing is key to improving the understanding of how nanomaterials physical and chemical characteristics affect their toxicity. A number of data repositories are filling this role, but there are still differences in what data each captures, and how the data is analyzed and interpreted. The National Institute for Occupational Safety and Health (NIOSH) is working on setting standards that will establish guidelines on what kind of information these data warehouses need to capture. Even as this underlying infrastructure is being built, it will not adequately represent the diversity of NPs in production until a systems approach to high throughput screening of nanomaterials is established.

Chapter 2 – Testing for Nanomaterial Induced Toxicological Response

Toxicological Model Options

In order to assess nanomaterial toxicity, an appropriate model must be established. There are many factors taken into consideration, such as whether to use cell culture or the whole animal to test nanotoxicity. Cell culture offers the ability to use multiple cell types and allows for rapid assessment. However, a major limitation of cell culture is that results are often not easily translated to human responses. While the most human-relevant model is a non-human primate, this carries two major limitations: (1) the amount of nanomaterials necessary to complete a study and (2) the inability to adapt the model system to a high throughput approach. In order to complete a primary toxicity study using any exposure scenario (dermal absorption, ingestion, or inhalation), large quantities of nanomaterials would be needed. This poses a difficulty for the manufacturers, due to the large expenditures necessary to generate the quantity of nanomaterials needed and also the fact that some can only produce very little material from the current method of synthesis. This system would also not be appropriate for high throughput screening due to the limited number of animals produced at one time, and the large husbandry costs. Both the rat and murine model would be an appropriate small, whole animal system alternative to non-human primates. However, these models possess the same limitations in regards to quantity of nanomaterials needed and the inability to conduct high throughput screening. An ideal model would be a whole vertebrate animal system capable of high fecundity, rapid external development, a sequenced and annotated genome, and a high degree of basic genetic similarity with humans. A suitable option is the zebrafish.

The Ideal Model - Zebrafish

Traditionally used as a model for studying the development of vertebrates, zebrafish are a small (about 3 cm in length) tropical teleost fish native to India. A single female can produce hundreds of eggs per week, which are externally fertilized and develop optically clear for the first 48 hours after fertilization, allowing for easy manipulation and observation. Embryos develop visible red blood cells by 24 hours post

fertilization (hpf), almost all their organs by 48 hpf, a full beating heart by 96 hpf and a complete immune system within 30 days. Zebrafish are also amenable to studies of behavioral changes resulting from exposure. As early as 24 hours post-fertilization, embryos display arrhythmic tail bending. Modulation of the tail bending frequency is readily observed in response to several different chemical exposures at concentrations far below what is needed to elicit other detectable developmental changes. Embryonic behavior is thus becoming an extremely sensitive endpoint for rapid throughput toxicology screening. Zebrafish also share remarkable similarity at the gene and biochemical levels and recapitulate numerous human diseases. This allows for remarkably informative translation from embryonic screening to likely human response/risk. Zebrafish have the ability to regenerate lost tissues and damaged organs including the heart and spinal cord and fins which provides another facet of disease and toxicology screening for compounds that both promote regeneration or inhibit it [26].

Animals are generally most sensitive to chemical insult during early development, which makes it the ideal life stage to conduct toxicological studies. At this early life stage, embryonic zebrafish serves as a good model system because they are vertebrates that share many cellular, anatomical, and physiological characteristics with other vertebrates. After which, zebrafish development resembles other vertebrates, such as humans, providing ample opportunity to conduct other translational studies into adulthood.

Zebrafish are diploid, like humans, making them much genetically tractable than most other model fish species that are tetraploid [27]. The zebrafish genome ranges from 40 to >80% similarity to humans, depending on the gene examined [28]. The genetic similarity to humans, allows mechanistic studies to be more reflective of the likely human mechanism, and thus more translatable. Using the zebrafish model will not only enable high throughput screening, on the order of hundreds of thousands of embryos and larvae per study, but will also be an efficient catalyst for highly focused toxicology research in higher vertebrates.

Current Zebrafish Applications

The Tanguay lab has a standard protocol utilizing a three-tier approach (as illustrated in Figure 2) to screen for nanotoxicity. Tier 1 is the rapid toxicity screening. Tier 2 is cellular toxicity and distribution. Tier 3 is molecular expression. This approach comprehensively assesses the risk associated with a nanomaterial.

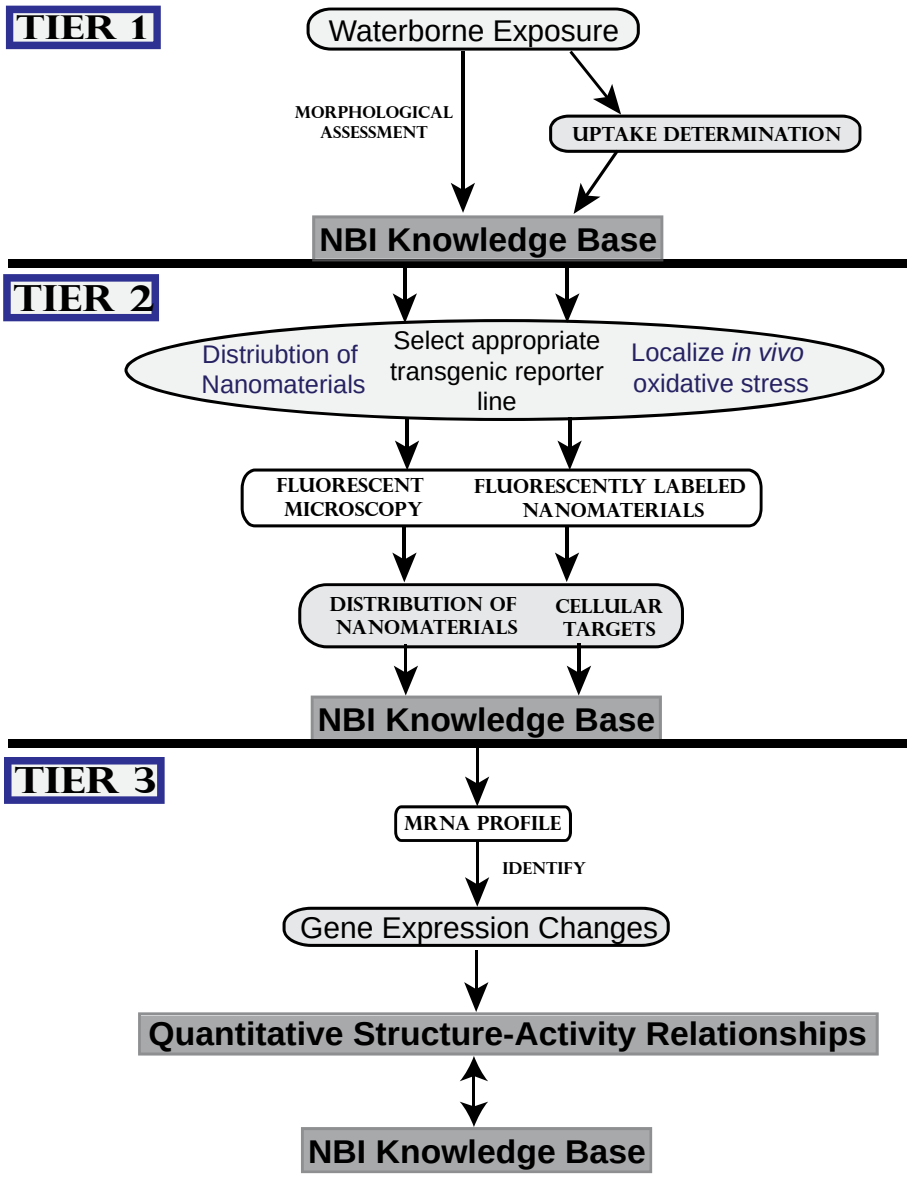


Figure 2.Overview of nanomaterial tiered experimental design schematic.
Tier 1 – Rapid Toxicity Screening. Tier 2 – Cellular Toxicity and Distribution. Tier 3 – Molecular Expression.

Tier 1 – Rapid Toxicity Screening

Rapid toxicity screening is the initial testing phase of any nanomaterial. This step is used to quickly ascertain if the nanomaterial induces adverse effects upon exposure to embryonic zebrafish. Adult zebrafish are group spawned, and embryos are collected and staged according to Kimmel et al (1995) [29]. The chorion is enzymatically removed at six hours post fertilization (hpf). The removal of the chorion helps to ensure nanomaterial uptake and bioavailability, as it acts as a protective barrier. Once the chorion has been removed, the embryos are kept in clean media and allowed to develop until eight hpf when the embryos are placed, one per well, into 96-well plates for exposure.

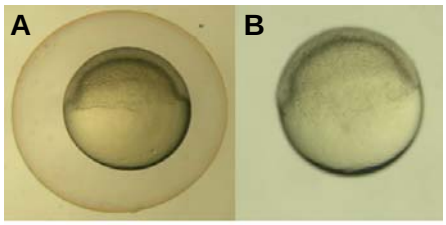


Figure 3. Six hours post fertilization embryos.
a) Six hpf embryo with its chorion.
b) Six hpf embryo after using pronase to enzymatically remove its chorion.

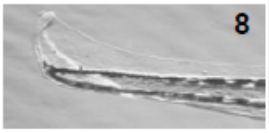
The 96-well plates are prepared with each plate representing a single nanomaterial. There are 12 embryos per concentration with a total of seven concentrations (fivefold dilution from 0.016 to 250 µg/mL) and a control (solvent vehicle or fish water). If a solvent is needed to aid the nanomaterials into solution, dimethyl sulfoxide (DMSO) is used. DMSO does not affect development of zebrafish when used at or below 0.1% final concentration [30-33]. Each well contains 100 µL of exposure solution. Embryos are statically exposed to nanomaterial solution (without renewal) from 6 hpf until 120 hpf. The embryos are inspected for viability, developmental progression, and spontaneous movement at 24 hpf. At 120 hpf, the zebrafish are assessed for 15 morphological responses (yolk sac edema, bent body axis, eye, snout, jaw, otic, pericardial edema, brain, somite, caudal fin, pectoral fin, circulation, pigmentation, trunk length, and swim bladder; Figure 3) and a behavioral response (touch response). For each endpoint, a binary score of normal or abnormal is recoded for each well. If there is greater than 10% mortality across the control wells, the entire plate is repeated. If any adverse effects are observed, the nanomaterial will advance to tier 2 testing.

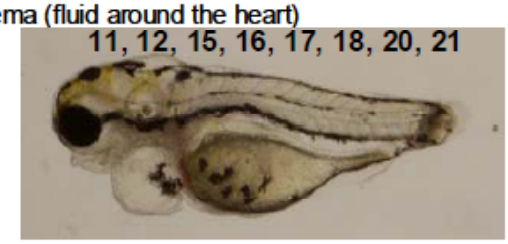
To determine if the nanomaterials that do not cause adverse effects are due to the nanomaterial not being uptaken, tissue uptake is measured. This is accomplished by using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS), which is designed to

measure trace metals. Embryos are exposed (as described above), and washed to remove nanomaterials that have adhered to the embryo, then digested with nitric acid. Once digested, samples are diluted and run on the ICP-MS. The measurements obtained provide insight to whether the nanomaterials were either taken up or tightly associated externally.

If it is concluded the lack of adverse effects observed is due to inability of the nanomaterial to enter the embryo, other routes of exposure are available to ensure bioavailability, such as injection of the materials directly into the embryo. Injections are done at six hpf with various concentrations of nanomaterials. Once injected, embryos are manually transferred into individual wells of a 96-well plate filled with 100 µL of embryo medium. Morphological and behavioral analysis as described above is completed, and tissue concentration is quantified to verify uptake.

1. **120 hr mortality** – dies between 24 and 120 hours post fertilization (hpf)
2. **24 hr mortality** – dies before 24 hpf
3. **24 hr sp. Mov** – no spontaneous movement at 24 hpf
4. **24 hr dev prog** - delayed development
5. **24 hr notochord** – notochord malformation (wavy notochord)
6. **axis** – curved or bent axis in either direction
7. **brain** - brain malformations or necrosis
8. **caudal fin** –malformed or missing
9. **circulation** – no circulation or blood flow
10. **eye** – eyes malformed, missing or smaller/larger than normal
11. **heart** – heart malformation, pericardial edema (fluid around the heart)
12. **jaw** – malformed
13. **otic** – malformed or missing
14. **pectoral fin** – malformed or missing
15. **pigmentation** – lack of pigmentation, overpigmentation
16. **snout** – shortened or malformed
17. **somite** – malformed or disorganized, missing somites
18. **swim bladder inflate** – failure of swim bladder to inflate
19. **touch response** – not responsive to touch at 120 hpf
20. **trunk** – short trunk, malformed or missing
21. **YSE** – yolk sac edema, swelling around the yolk sac





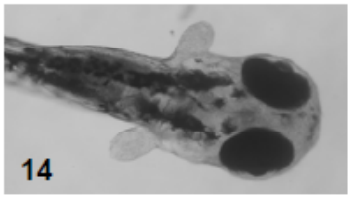


Figure 4. Visual Assessment of Zebrafish Morphology.
Images are given as examples of typical chemical-induced malformations observed in the zebrafish.

Tier 2 – Cellular Toxicity and Distribution

If a nanomaterial that has undergone tier 1 (rapid screening) is found to induce adverse effects, additional assays are carried out to determine whether it can cause other undesired effects. These assays are designed to investigate, at the cellular level, what type of interactions the nanomaterials are having with the biological system. The main techniques that are used for this tier are transgenic zebrafish and special fluorescent dyes which identify changes in the cellular oxidation environment.

There are many different types of transgenic fish [34], depending on what the desired endpoint is. Transgenic zebrafish can be used to monitor specific organs or certain functions. For example, if it is thought that a nanomaterial disrupted a specific cell population, a transgenic fish designed to have a fluorescent tag added to their DNA corresponding to that specific cell population is utilized to understand the nanomaterials influence. In transgenic zebrafish, when a pathway is activated, the area designed to glow (due to the fluorescent tag) will glow normally, which indicates normal activity. However, when exposed to a nanomaterial that causes damage to this area, the glowing will stop, indicating to the researcher that the nanomaterials are influencing that specific portion of the system. Images can be taken of this phenomenon by taking exposed embryos (as described in tier 1), mounting them onto glass cover slides, and imaging them on an inverted microscope to capture the fluorescent proteins. This is a powerful tool to enable determination of which cells within the zebrafish are being disrupted by the nanomaterial.

Cellular Oxidation

Cellular oxidation can also be detected by using a cellular death assay that can track occurrence of cellular death through whole embryos by use of specific stain, acridine orange (**Figure 4)**. To conduct this test, embryos are exposed to nanomaterials in the same manner as described above in tier one. After a short exposure, embryos are removed from the solution and incubated with the acridine orange stain which will label cells that have lost membrane

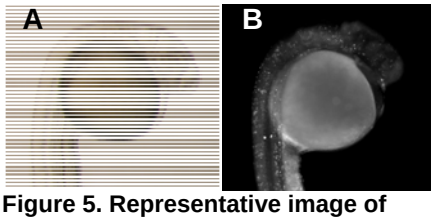


Figure 5. Representative image of cellular death occuring in embryonic zebrafish.
(A) A brightfield image of a 24 hpf embryo. (B). An image demonstrating cellular death occurring (white specks)

integrity [33]. This process is used to locate and quantify cell death in embryonic zebrafish.

Distribution

To determine the nanomaterial distribution fluorescently tagged nanomaterials are used (quantum dot labeled). Once the embryonic zebrafish has been exposed to the nanomaterial, fluorescent microscopy can be used to determine where the nanomaterials have distributed. One consideration is that the fluorescent tag could change the behavior of the nanomaterial. Another localization method is to fix and section embryos then analyze using a scanning or transmission electron microscope individually for each section of the entire animal. Software is then used to reconstruct the animal in three dimensions from the images obtained from the slices.

Tier 3 – Molecular Expression

With the knowledge gained from tier 1 and 2 about how the nanomaterial is affecting the biological system, a gene expression study is conducted to understand this interaction and its molecular targets. A reproducible endpoint such as the adverse effect observed in tier one is used to anchor the molecular studies.

Window of Sensitivity

The first step is to determine the exposure timing needed to induce the reference endpoint in the embryonic zebrafish (window of sensitivity). The window of sensitivity is the period during embryonic development that the nanomaterial influences the regulatory pathways that presumably elicit the phenotypic change. 24 hpf zebrafish embryos are exposed to nanomaterials for a range of times by either delaying the exposure start time, or stopping the exposures earlier. Only one of these manipulations is done at a time to determine what the optimal period and duration the nanomaterial has to be present to produce the endpoint.

Gene Expression

Knowing the window of sensitivity sets the stage to complete a gene expression study to understand what genes the nanomaterial is directly affecting. Forty embryos, three replicates of 10 and a control set of 10, are exposed to the nanomaterial over the window of sensitivity. If the window of sensitivity is after 24 hpf, there are additional

difficulties in narrowing down the gene expression linked to the endpoint because there is an increased amount of natural gene expression changes associated with this stage of development which can mask the expression changes induced by the nanomaterial. After static exposure until the end of the window of sensitivity, the embryos are collected at time points and washed and RNA is isolated. Samples are then processed and undergo bioinformatic analysis to investigate how the nanomaterial exposure affected the global gene expression.

With the knowledge of where the nanomaterials are localizing, researchers can correlate the known function of the genes with altered expression with the tissue types known to be the localization targets of the nanomaterial. This provides a basis for cell pathways affected and an initial mechanism for how the nanomaterial induce an adverse effect.

The Need for High-Throughput

With the number of novel nanomaterials being produced, with varying size, shape, chemistry, and surface functionalization, it is critical that a system be implemented that enables the very robust, single pass toxicity screening of hundreds or thousands of nanomaterials prior to use in consumer products. Due to the nature of this emerging field, the toxicological screening must be completed quickly and thoroughly to avoid delay in implementing new technology as well as further pursuit of “bad” leads. In addition to simply screening the new nanomaterials, an infrastructure is needed to rapidly screen nanomaterials that currently exist, but have little or no toxicological data available.

To have a successful system, the following characteristic must be possessed:

- Both time and cost efficient
- Reliability and reproducibility
- High capacity to screen large libraries of nanomaterials

In addition to the physical handling of the nanomaterial samples, a database must be implemented to collate and maintain records of toxicological results to enable online searches of what key physico-chemical characteristics are associated with particular classes of nanomaterial. Both speed and cost-efficiency and information content of the

screening process and results are critical to acceptance by industry. Industry is driven by the potential revenue from products containing nanomaterials, so to encourage cooperation from them, the test cannot be high cost or cause delay to the incorporation into their products. In order to succeed in implementing a nanomaterials toxicity-screening program, the industry sector must agree to adopt this process and participate. Without their cooperation, industrial nanotechnology development is compromised. It is a shared public/private sector responsibility to understand nanomaterial safety. Efforts must be taken to prevent development of nanomaterials that recapitulate past blunders of both the chemical industry and regulatory agencies.

Toxicology screening for nanomaterials must be robust, reliable, and repeatable. It is challenging to implement *de novo* as it requires highly trained laboratory technicians, computer programming, and sophisticated instrumentation Laboratory technicians are not an easy or inexpensive resource to obtain. Being a human resource, maintaining consistent and systematic methodology is difficult, which may prevent reproducible results. The data generated will be massive; therefore, a curated database must be established.

One drawback of having laboratory technicians is the cost of acquisition and training. These positions require a person with an appropriate education, and either someone with an existing skill set or someone who would need extensive training. These are both time and money intensive. In addition, laboratory technicians are full time staff, which prevents intermittent employment, thereby causing a constant cost to the system. This constant load is not beneficial for a company.

Another issue is the difficulty of obtaining consistency among laboratory technicians. This variation will result in decreased reliability across the screening system. In addition, the use of humans for these highly repetitive processes can lead to complacency and mistakes. The tedious nature of this process is a major factor in repetitive stress injuries, worker boredom and errors. Constant laboratory technician replacements are unavoidable and thus result in varying procedural changes over time as tribal knowledge is passed along. All of these are major obstacles that must be overcome if human laboratory technicians are to be utilized.

The automation alternative

Automated robotics are perfectly suited for highly repetitive tasks. With robotics, a large initial monetary investment is incurred, but the operating costs for robotic work cells are minimal, due mainly to the power and consumables that they utilize. Work cell maintenance is minimal, as most of the industrial systems are designed to be maintenance free for a minimum of 10 years. Robotic work cells can also be idled without the need to lay off workers or incur other expenses. Scaling up capacity is also straightforward; simply increase the number of work cells to match the needed capacity.

Automating processes also provides other benefits. By utilizing automation, process variation between work cells (analogous to that between laboratory technicians) is almost nonexistent. In addition, the process is consistent over time; the work cell will perform precisely the same regardless of its age. This effectively eliminates variability encountered amongst workers over time. This is extremely valuable to a process that needs to be reliable and repeatable such as nanomaterials screening.

Data collection and storage is also simplified by using automated processes. Creating an automated system utilizing machine vision to analyze and collect data, eliminates human interpretation. The data analysis can occur simultaneously and systematically. This will remove the possibility of data entry errors since data is captured and stored directly into the database.

To obtain the benefits of utilizing automation, there are obstacles that need to be resolved prior to implementation for nanomaterial toxicity screening. Unlike a manufacturing environment where automation is common, there is a lot of variability in a laboratory setting, especially when living organisms are involved. This complicates the automation process, as the system cannot depend on materials being in a known location.

Additionally, embryonic zebrafish are living organisms that move, which causes the robotic automation system handling problems. In industrial settings, handling non-living materials is relatively easy. However, since zebrafish embryos are small and fragile, this presents a hurdle to develop a method which can reliably manipulate them without causing any adverse effects.

Challenges with the model

Injections of nanomaterials into zebrafish embryos are routinely conducted by humans for toxicological studies. But this is an automation challenge to address that will have a huge pay-off in efficiency and reproducibility. There are several ways that have been reported as to how this could be accomplished, but none has been demonstrated in a functioning high throughput environment.

Chapter 3 – Automation Design Options

The Tanguay lab had previously invested in some automated screening equipment at the outset of the author's study. The device that was selected was a high content imager shown in Figure 6. This instrument is a $250K, state-of-the-art platform for the automated capture of high resolution images of micro and macro subjects in many formats. The high content imager is an endpoint platform in that it does not contribute to the automation of previous screening steps, only the final data collection.

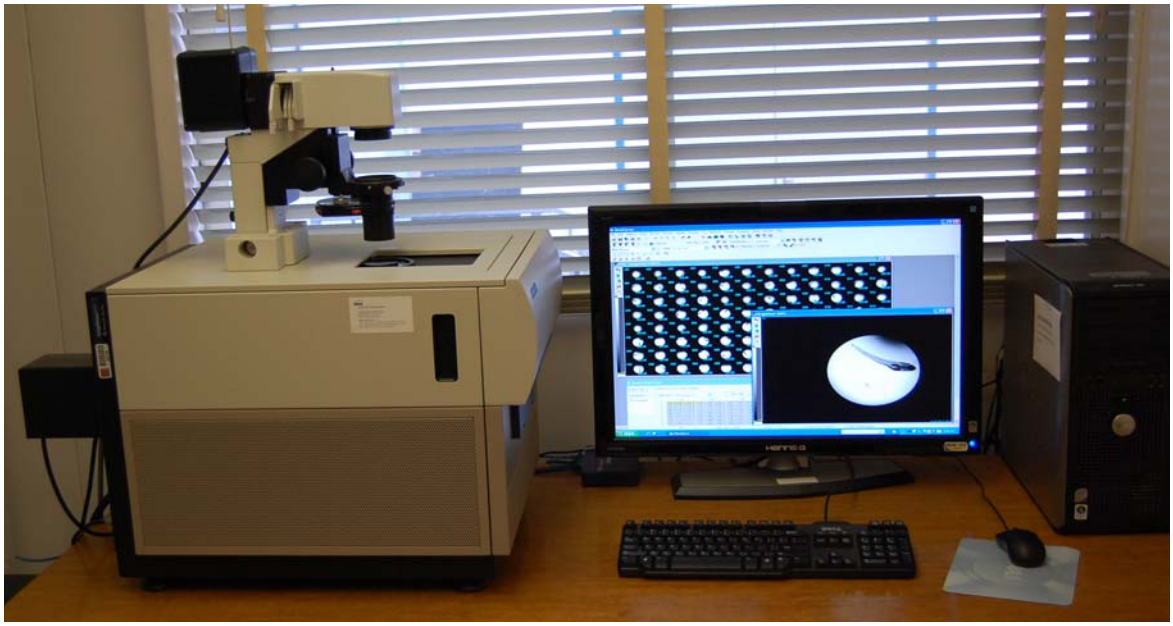


Figure 6 - High Content Imager.
A MDS High Content Imager designed to analyze larval zebrafish for morphological malformations.

Integrated automation of the platforms for screening steps upstream of imaging was the critical challenge that the author addressed for his thesis. Several technologies were identified to assist with these steps. Ultimately, the mundane task of having to create dosing solutions at seven different concentrations per nanomaterial was addressed with a robotic liquid handler fluid handler from Caliper Life Sciences. With both the creation of dosing plates and analysis of the zebrafish configured for high throughput, the last major obstacle remaining was placing individual zebrafish eggs into the wells of a plate pre-filled with dosing solutions dispensed by the fluid handler.

Automated Embryo Placement

Although it may look simple, placing zebrafish embryos into the individual wells of 96-well plates is a challenge. Several factors pose challenges to automated manipulations of the embryos. Embryos are living organisms, and quite fragile, which limits what materials can be used to handle them, in addition to the amount of force used to move them.

Possible System Directions

To successfully pick up and place zebrafish embryos, microfluidics, shaker table, mechanical and micropipettes systems were each considered.

Microfluidic System

Microfluidics is a promising field for many applications. In theory, a microfluidics system could potentially incorporate several key features into one unit making dilutions of the material to be tested as well as the embryo media that zebrafish embryos are kept in. Such a system could also move embryos in solution through a circuit-like decision tree to filter out poor quality or dead ones from good embryos and then place them into the sample solution that was diluted and mixed with embryo media earlier. This eliminates the need for another system entirely while maintaining a very small footprint. A recent example of microfluidics utility in this area is report of embryo injection using microfluidics with electroosmotic dosage [35].

Microfluidic Prototypes

Several prototypes and proofs of concept were built to determine if a microfluidics approach would be feasible for this application of high throughput screening with embryonic zebrafish. The first prototype, a slug generator, was built (Figure 7) by manufacturing slugs of solution interspaced with air. This device was made by milling a channel and other features for valves and connections into a polycarbonate top and bottom piece that was bolted tightly together to form a closed channel. A modified SMC air pilot valve was used to control pressurized air that would enter the microchannel and form the air slug. This device worked well, except that the required cross section of the channel allowed the fluid to behave with macrofluidic properties, which compromised the integrity of the slugs by allowing cross talk (contaminated fluids and residues which

move between the slugs of fluid) as they moved throughout the system which leads to contamination of samples as well as varied slug volumes.

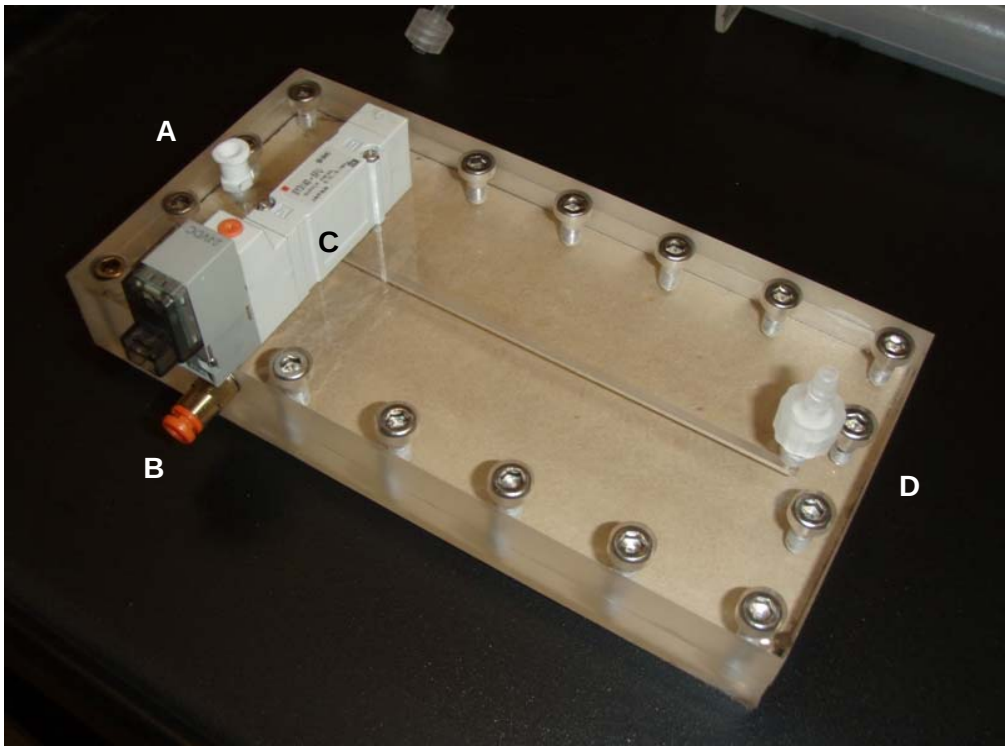


Figure 7 - Microfluidic Slug Generator
A) Fluid enters the channel at the left port. B) Air enters through a port and flows into the valve. C) The valve injects air into the fluid stream which is broken into slugs. D) Fluid broken into slugs and separated by gas exit through the right port.

In order to ensure that *micro*fluidic flow behavior was achieved, the next generation devices constructed had circular channels in order to reduce the total cross-sectional area of the channel. In order to obtain round channels, drills were utilized, but this limited the overall length of channels to less than two inches, which is the maximum length which could be accurately drilled with small drill bits. To overcome this limitation, tubing was substituted for extended distance runs as its round shape would preserve slug flow. At interconnections or points of automation, small microfluidic chips would be utilized. An example a prototype to address the problem of macrofluidic air and liquid slug behavior is illustrated in Figure 8 - Microfluidic Zebrafish Component

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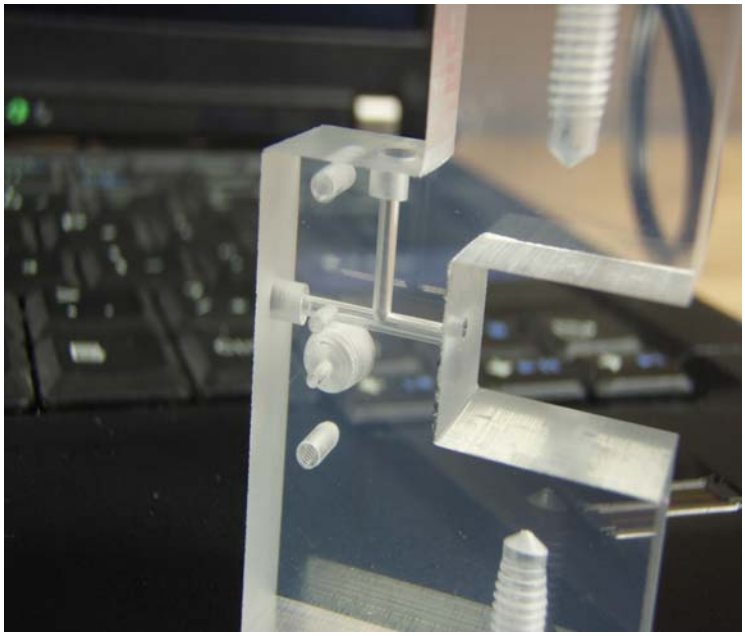

Figure 8 - Microfluidic Zebrafish Component
Microfluidic component of Figure 9 showing the three short channels (center) which were drilled to enable slug flow of embryo media and embryos through the device.

The part shown in Figure 7 was part of a larger system that was designed to generate slugs and simultaneously insert eggs into them, as shown in Figure 10. The microfluidic segments all utilized circular channels manufactured using drill bits. This device suffered from issues relating to the polymer materials used. Proper slug flow can be achieved, but once the eggs were introduced into the system, they became stuck to the polymer material and were damaged. No solution could be found to prevent the embryos from sticking to the polymer.

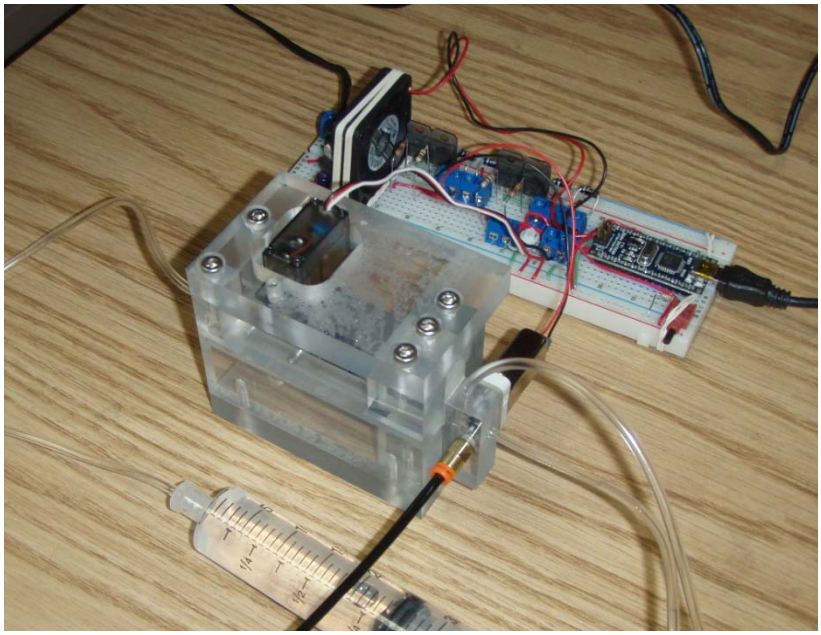

Figure 9 - Microfluidic Zebrafish System
Fully assembled system utilizing microfluidic chip illustrated in Figure 8 and electronic control circuitry for generating slugs and inserting embryos.

Limitations of Microfluidics

Although there are positive attributes to this technology, the embryo size was almost too large to be manipulated in microfluidic channels, thereby requiring a channel size between the boundaries of microfluidic and macrofluidic behavior. Second, the polymer platforms (polycarbonate and acrylic) that are routinely used for microfluidics because of the ease and cost to manufacture are not compatible with the zebrafish embryos causing them to either stick to the surface, or get stuck in the channels, and become damaged.

The materials compatibility issue could potentially be solved with another material, but it was determined that searching for and evaluating the other materials would not likely be achieved in an acceptable time frame.

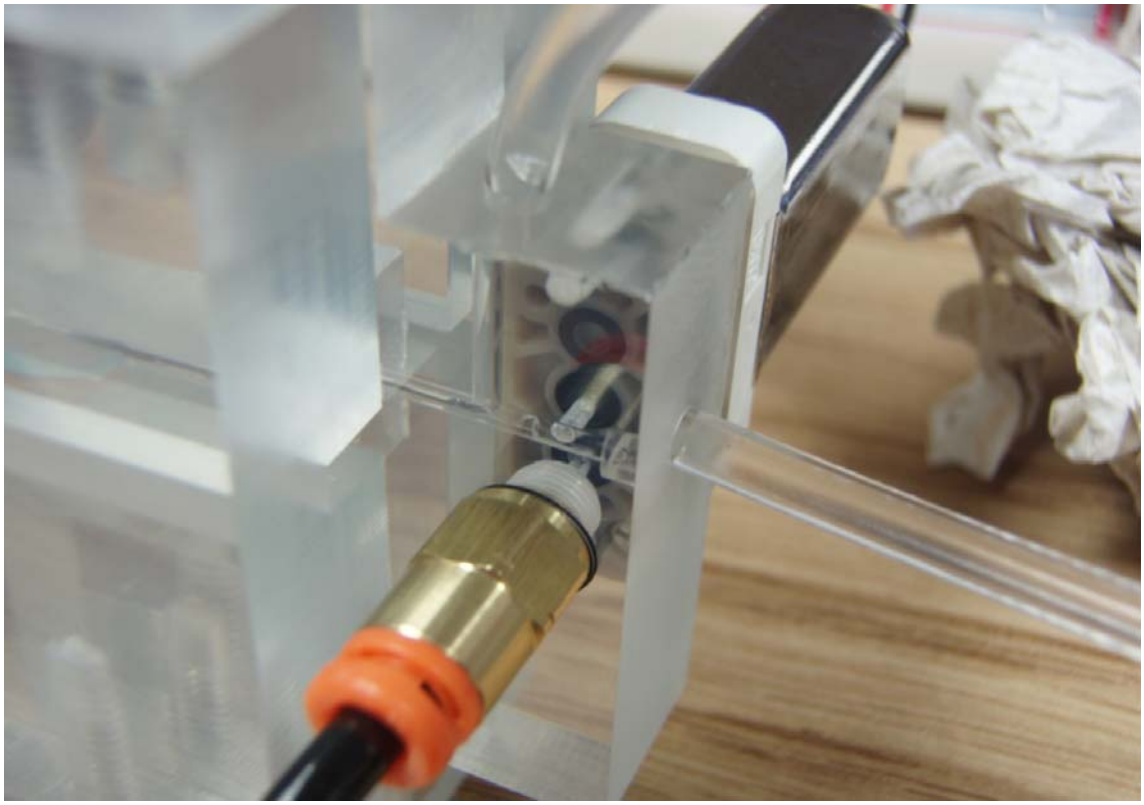

Figure 10 - Close up of microfluidic zebrafish system

Shaker Table Design

Shaker tables have been utilized for years in commercial industry. They are essentially a vibrating surface that causes parts to move around. If the shaker tables' purpose is to move parts, the entire surface can be sloped causing the parts to move in the direction of the slope. If the purpose is to position parts, features such as ridges or holes can be utilized which the parts move around until they are jostled into position.

Implementation

The next approach tried was a shaker table to move embryos around in solution over a pattern of detents. The negative buoyancy of the zebrafish embryos caused them to settle into the detents in the shaker table. Each detent is large enough for one embryo, and once all the detents were full, the remaining ones were flushed off of the shaker table. A sliding bottom is moved to allow the eggs in the detents to pass through to a 96-well plate below it.

Limitations

This system worked as expected, placing embryos into the detents during the loading stage. Unfortunately the embryos did not drop out of the system as expected because they were held in place by the surface tension of the water. With a light agitation, embryos would locate themselves correctly in the detents; however, the buoyancy of embryos and water were similar such that moderate movement would wash them back out. When the extra embryos were washed off the shaker table, approximately 20% of embryos in their detents were lost with it.

Due to the problems associated with containing the embryos in the detents, the shaker table was not an appropriate design for high throughput, since it would require more labor to put the embryos back into the detents each time.

Mechanical Grabber System

The most common form of manipulation in an automated work cell is the mechanical pneumatic or electrical gripper designed to grab parts (Figure 11). Pneumatic grippers are available in a wide variety of sizes capable of gripping objects weighing several hundred pounds to manipulating 500 µm grass seeds. Mechanical grippers provide a good means of positive control of parts that need to be picked up and placed accurately.

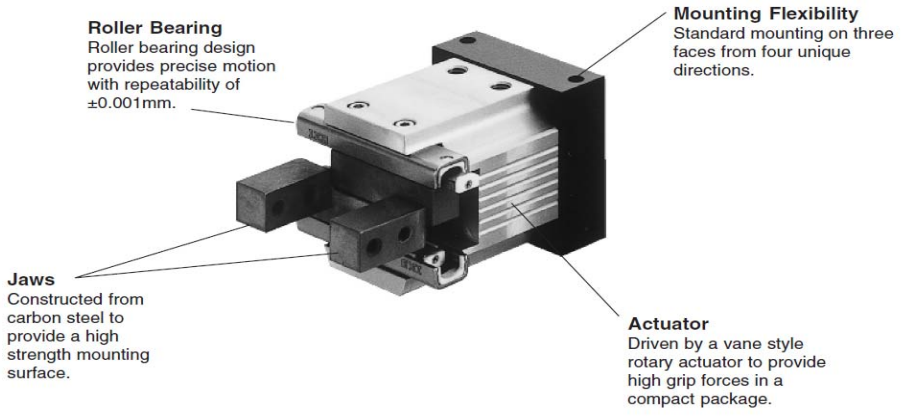

Figure 11 - Parker Pneumatic Gripper.
Image courtesy of Parker

Limitations

A mechanical gripper possesses limitations when used on living organisms such as embryonic zebrafish. First, the eggs are not a solid object; they are soft and fragile, and are easily damaged when too much force is applied. Force control of mechanical manipulators in not an easy or an economical task. The force range needed for handling zebrafish eggs is so small that the gripper control would be problematic. In additional testing, it was also determined that the eggs would most likely not drop free when released. Surface tension of water held them to a flat surface, meaning that the eggs could not be reliably released by the gripper. The mechanical gripper option was determined to be impractical for these reasons.

Glass Pipette System

Glass micropipettes are traditionally used in the laboratory to manipulate embryos. Silica glass has been shown to not adhere to the embryos, which can damage them. They also allow precise control over fluid that is picked up and dispensed due to their small diameter which can be selected to draw the embryo inside or have it held on the tip for microinjections or other manipulations where access to the embryo is needed.

Implementation

The last system evaluated was based on using a micropipette. This system most closely mimicked the human handling technique and, as a result, was thought to have a high probability of success. A micropipette does not involve any new forces or stresses on the eggs that could affect the data generated.

This process was tested utilizing a manual micropipette to simulate picking up and placing embryos in a manner similar that of a robot manipulator. We were able to realistically simulate the actual process without acquiring a robot by building a prototype end effector and manipulating it manually by hand. We demonstrated that the micropipette could pick the egg up and place it in the well reliably without damage to the egg, thus we could incorporate a robotic arm to transport the egg to the desired location.

Limitations

There are few limitations to this system; it is very similar to the current processes that are used. The largest issues that will be encountered are manufacturing the glass micropipettes. There are not many custom manufacturers who can produce custom

glass work and their services are often cost prohibitive. Simplicity of design to allow in house manufacturing will be important.

System of Choice

After analyzing the positive and negative traits of each system listed above, it was determined that a system utilizing a glass micropipette would have the highest likelihood of success.

- Microfluidics
    - Difficult to manufacture
    - Material incompatibilities.
- Shaker Table
    - Embryos too buoyant
    - Inconsistent placement of embryos
    - Surface tension prevented embryos from dropping free
- Mechanical Gripper
    - Unable to reliably pick embryos up without damage
    - Unable to drop embryos due to surface tension
- Glass Micropipette
    - Similar to current process that is proven
    - Will work with commercially available automation equipment
    - Considerations for in house manufacture

Chapter 4 – Selected System Design

After considering the above options, it was determined that a solution employing a micropipette manipulated by a robotic automation system would be the system with the highest likelihood of success for this project. This system leverages existing, well developed and understood technology for the automation aspects of the system, while relying on a mechanical process that is almost identical to current practice for picking up and manipulating the embryos. This selected system has four main components: the robotic manipulator, a machine vision system, a micropipette end effector with a pump to actuate the micropipette, and a work cell to enclose all of these systems.

Selected System Design

Robot

For the initial trials of this system, an 850mm reach, six axis industrial robot from Denso Robotics (Long Beach CA.) model VS-6577-GM was selected to be the robotic manipulator. This robot offered the best combination of size, speed, and precision to support a broad range of other automation development in the lab. Since the ultimate capability requirements for the system are undefined, a more flexible robot was an attractive option as it allows for many approaches to be tested with an investment in a single platform.

The absolute maximum that the robot can reach is 934mm from the center of the base. The maximum composite speed of the robot end effector is 7600mm/second (7.6 meters/second). This model is a six-axis robot, meaning that it has 6 joints that allow the end effector to move with six degrees of freedom. This allows the robot to position the end effector in any orientation and to reach into or around obstacles and is ideal for complex maneuvers, such as injecting zebrafish embryos, another goal of this project.

This Denso robot system is composed of three major components as shown in Figure 12; the robot, the controller, and the pendant. The controller is the brain of the robot. It houses the computer that controls the robot, as well as the transformers, amplifiers, motor controllers, and I/O circuitry. Since the work cells will be relatively simple, the controller will be used to control the entire work cell. The pendant is the basic user interface into the controller. This enables basic jogging, or moving, of the

robot by hand. It allows users to create, edit and run basic programs. The robot is the component of the system that actually moves. It consists of motors and sensors that are all tied directly back to the controller.

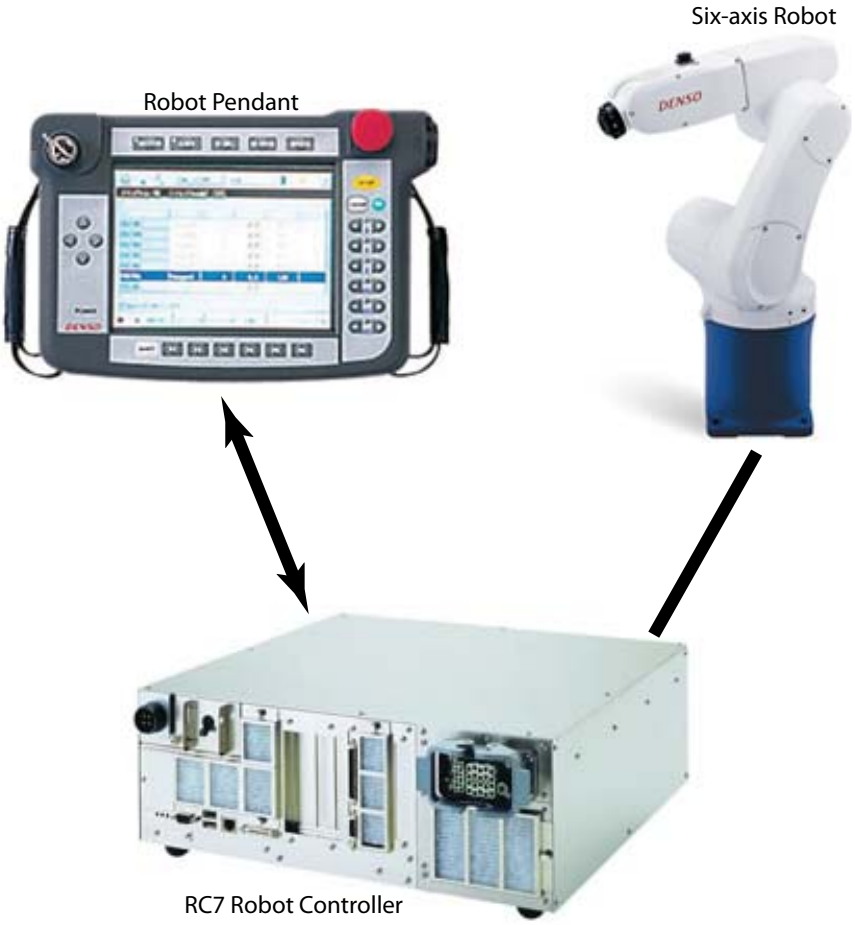

Figure 12 - Denso robot components.
This image illustrates the three major components of the robotic system sold by Denso, the controller, robot, and pendant.

Vision System

The key requirement of this automation project is that it must be able to adapt to the inherent variance of working in a lab with living organisms. This requirement is addressed very effectively by modern machine vision systems. Machine vision is a generic term used to denote a camera system with image processing software that enables automation systems to analyze and interpret the visual world in which they function. Cognex (Natick, MA) machine vision cameras were selected for this project

due to their computational power and best-in-class vision software capabilities, which we believe offers the highest probability of success. Cognex cameras also support communication with the robot controller and other components of the system through Ethernet, serial, and other standard industrial communications protocols. This offers key advantages in the selection and implementation of custom components such as the end effector and micropipette pump.

The machine vision system itself consists of three main components; the camera, the lens, and a power supply. The camera contains both the imaging sensor and the processer that analyzes image content and communicates with other system external components. The lens can be selected from any number of manufacturers, styles, and sizes to best suit the task. The camera power supply is located remotely with all power passed via the Ethernet line going to the camera using Power over Ethernet (PoE).

For this work cell, the machine vision system will identify and locate individual zebrafish eggs. Since one of the key principals of this system is flexibility, instead of a permanent mount for the camera above a set work area, it was decided to mount the camera onto the robot end effector itself. This mounting location has both advantages and disadvantages. The main disadvantage of the camera being mounted on the moving end effector is that it makes the location of a part in the camera's field of view difficult to determine in the robot's coordinate system, because the camera's reference plane is constantly moving in relation to the robot's coordinate system. The advantage of mounting the camera on the robot end effector is that it affords unparalleled flexibility in where the robot can "see" as one camera can be used to view multiple areas. In addition, during design changes, instead of having to modify existing fixtures or build new ones to position the camera in a desired location, the robot program can be quickly and easily modified to position the camera in any position desired. The author found the flexibility of this mounting location key to the efficient development of the vision software algorithms.

The main functionality of the machine vision system is to locate zebrafish embryos in a shallow dish of bulk solution. One of the key software capabilities motivating selection of Cognex machine vision system was a feature called PatMax. This is a special vision algorithm, at which Cognex excels, that helps with locating complicated and low contrast parts. Machine vision systems can easily detect very

small components, but they generally rely on a large contrast between the part and the background to find an “outline” of the part, or simply determine the part’s edges.

One of the largest challenges facing the zebrafish vision system was the differentiation of the embryos from the background. Contrast can be optimized with several lighting setups such as back lighting, side lighting, and directional lighting from the top. Backlighting is a traditional means to increase contrast in machine vision illumination systems, and was used to obtain the high contrast in Figure 13. Backlighting did not provide optimal results in the case of zebrafish embryos because they are essentially translucent at the stage of development during which they are manipulated.

Figure 13 – Example of a high contrast part (gasket).
This image illustrates a typical level of contrast suitable for machine vision systems. *Courtesy of Cognex*.

Once the zebrafish embryos had a high enough contrast to be detected by the cameras, programming the cameras to reliably detect individual embryos began. The Cognex PatMax algorithm was key to implementation of this capability. This pattern software not only detects the external edges of the eggs, but also internal features, something that could not be done with other systems requiring much higher contrast. Once a “pattern” for the embryo’s features was developed, the camera locates all objects in the field of view with that “pattern” and through this process identifies the embryos. Once an embryo has been located, the center is determined in pixel coordinates (the camera coordinate system), which are then translated into the robot coordinate system.

The camera is always the same distance from the embryos when an image is taken. This equates to each pixel being equivalent to a certain distance in the real world. Through a calibration process, this distance is determined, and then normalized to the center of the camera’s image. When an image is taken, its precise distance on

the plate bottom in millimeters from the center pixel can be ascertained. When the robot pauses to take a picture, it also passes the camera's current X and Y coordinate in millimeters to the camera. This value is then used to convert the actual location of the embryo into the robot's coordinate system; which is passed back to the robot controller, providing the robot controller with a precise location of an embryo.

In addition to determining the exact location of the zebrafish embryo, the camera also attempts to detect if the embryo selected has an advanced state of necrosis. This is done by finding the edge of the embryo, then looking at each pixel value that makes up the bulk of the embryo. If the embryo is dead and necrosis has set in, it becomes opaque and white, which glows much brighter than the living embryos. This manifests itself in the image by having a higher number of whiter pixels as shown in Figure 14. The average pixel "whiteness" is compared to a threshold value, if it exceeds the threshold the embryo is considered dead and this information is passed along with the location to the robot which will pick up the embryo and discard it to a waste bin.

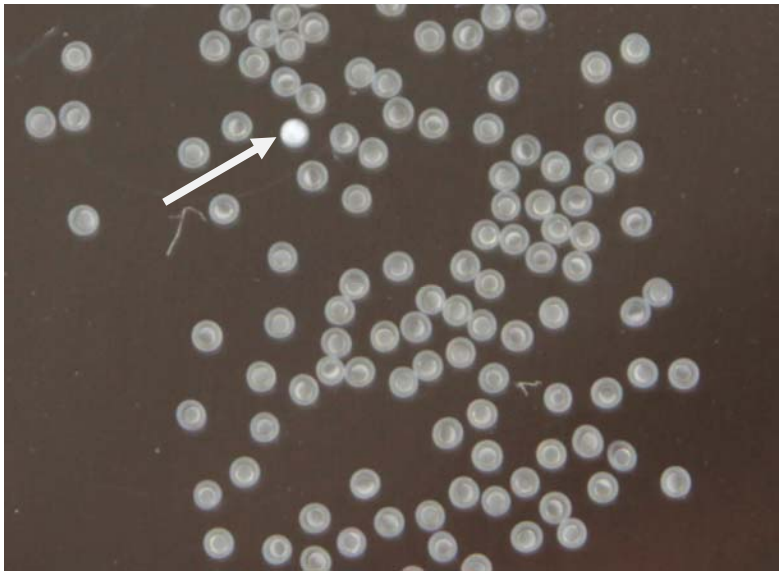

Figure 14 - Necrotic embryos.
This image illustrates the contrast between normal embryos and a necrotic embryo (indicated by arrow).

End Effector

The selected design utilizes a glass micropipette on the end effector to pick up and manipulate zebrafish embryos. The pipette was manufactured from simple silica glass, inert to zebrafish embryos. It is also available in a number of diameters, which provided flexibility during system development for optimizing embryo pickup. Embryos could be completely drawn inside a larger diameter glass tube, or with a smaller diameter tube, they could be drawn up against the tip and held in place with suction. This external suction hold was ideal because it allowed visual inspection of an embryo under a microscope by a separate machine vision camera. This made possible the automation of microinjection (nano-liter quantities) of embryos with test reagents.

Pump Backend

The end effector micropipette is connected to an actuator that enables the robot to draw up air/fluid to pick up the embryo, and then to expel the fluid to dispense the embryo. Several options were considered, including modifying conventional manual pipettes and commercial syringe pumps. After experimenting with both, it was determined that more precise control and the ability to vary the process parameters were required. An electronically actuated solution with a high level of precision in the control of the amount and speed of the fluid draw was essential, which necessitated the design and fabrication of a custom system.

In order to achieve these objectives, commercial equipment was purchased and combined to produce a pump base that had the speed and precision needed. These components came from OEM Dynamics and Animatics (Santa Clara, CA). The backbone of the pump is a heavy VL series stage from OEM Dynamics. It is equipped with a 100mm travel lead screw that moved the stage 6mm for every revolution. The motor is a NEMA 17 smart motor from Animatics. This is a servo motor that enabled high speeds, high precision, and theoretically infinite positioning. It is equipped with a 4000 count encoder, providing 0.009 degree positioning accuracy, which provides the stage with 0.0015mm, or 1.5 micron positioning. With the stage, couplings, and all components considered, the stage had approximately 10 micron uni-directional precision and 20 micron bi-directional precision from prior experience. The stage is rated at 500mm per second, allowing the entire length of the actuator to be traversed in under 0.25 seconds.

Assuming that the stage is able to obtain 10 micron linear precision, the diameter of the syringe used will determine the volumetric precision. For example, if a Hamilton gas tight syringe is used that has a total volume of 50ul over a travel of 60mm, then the total volume per mm is 0.83ul. With the linear positioning of the system estimated to be 10um, the estimated precision will be 0.0083ul, which is more than adequate for the requirements of the system.

Graphical User Interface

The graphical user interface (GUI) allows users to easily interact with and control the system. Instead of users having to utilize the low level command line commands that the robot controller uses to function, the GUI presents the user with an easy to understand visual control mechanism to operate the work cell. When a user interacts with the GUI it acts as a translator issuing commands to the robot controller over Ethernet in command line controls that robot understands. This is an important feature to allow those with minimal or no computer training to operate the work cell safely and efficiently.

The GUI software is implemented in Microsoft Visual Basic 2008 and is running on a Dell PC computer. The user interface is through an LCD Elo touch screen that utilizes Surface Acoustic Wave (SAW) touch detection technology. SAW technology utilizes a solid glass screen that is responsive to inputs from any soft object such as a finger (including when wearing gloves) or an eraser. The SAW touch screens do not need to be calibrated, and do not “drift” with time, making them an ideal selection for robust and low maintenance input devices.

The GUI computer is be mounted inside the frame of the work cell with the touch screen outside the robot’s work envelope so users may interact with it when the work cell is in use. By utilizing a touch screen there is no need for a mouse or keyboard to make inputs to the system. Industrial automation applications typically use touch screens because they provide a straightforward and intuitive operator interface, which reduces training and assures more reliable operator interactions with the system.

A preliminary design for the GUI layout is shown in Figure 15. The GUI has the basic and most common controls arranged in an organized way on the main screen. Advanced controls that are not used by operators are placed on a screen that is not

accessible by operators to prevent unauthorized changes and damage to the system. Some of the important aspects of the GUI are:

- Start, Stop & Pause: Initiates the robot process as selected in the process configuration section, and pauses or stops the process if running.
- Speed: Controls the robot speed for performing the process. This is important for different combinations of process steps may require slower robot speeds for reliable handling.
- Process Configuration: This allows the operator to select the desired actions and plate configuration to determine robot's actions.
- Camera Images: As the cameras acquire images, they are displayed for operator monitoring.
- Status: Provides basic status of system connections and operational status.
- Advanced Controls: Password protected screen to restrict operators from changing the advanced configuration of the system.

Figure 15 - Screen shot of Graphic User Interface

System Integration

When fully integrated, this system will locate and identify zebrafish embryos, pick up individual embryos, transport them to another location, and deposit them precisely where desired, with no errors. This offers an effective high throughput solution for automated pick and place of zebrafish embryos from bulk solution to 96-well plates.

Chapter 5 – Results and Discussion

The goal of the first prototype system was to provide a platform for researching what a system could do, and the first implementation build was designed to provide a flexible platform. Starting out, there were a number of requirements for the system being designed:

Requirement	Target	Acceptable
Picking up embryos	100%	95%
Putting embryos into 96 well plates without damage	100%	95%
Moving embryos with as little embryo solution (buffer) as possible	0uL	50uL
Detecting embryos with visible necrosis	100%	95%
Visual inspection of embryos	100%	90%
Injection of embryos	100%	95%
Precision dispensing of fluid	1uL	10uL

The implementation consisted of initial setup and learning with the Denso robot, design and construction of a flexible work cell, and integration of a machine vision system from Cognex. Finally, all components were integrated and tested.

Implementation

Phase 1 – Robot Setup

The Denso robot was received July 10th 2009. Once unpacked, the robot was initially setup while bolted to the skid that it was shipped on. This configuration allowed the robot to be setup and calibrated without other important components that were not yet received. Initially the system was powered with 120VAC which allowed the robot to operate in a reduced capacity. The system required a minimum of 240VAC for proper function.

With the Denso robot controller powered and the robot itself able to move at slow speeds the author began the process of learning the programming language, WINCAPSIII. As the basic coding and motion control was explored, the fundamental building blocks were mastered for all future programs to be built. Simple moves, taught points, and work planes were experimented to also master the manual operation and basic coding of the system. Figure 16 – shows the robot still mounted to the skid that it was shipped.

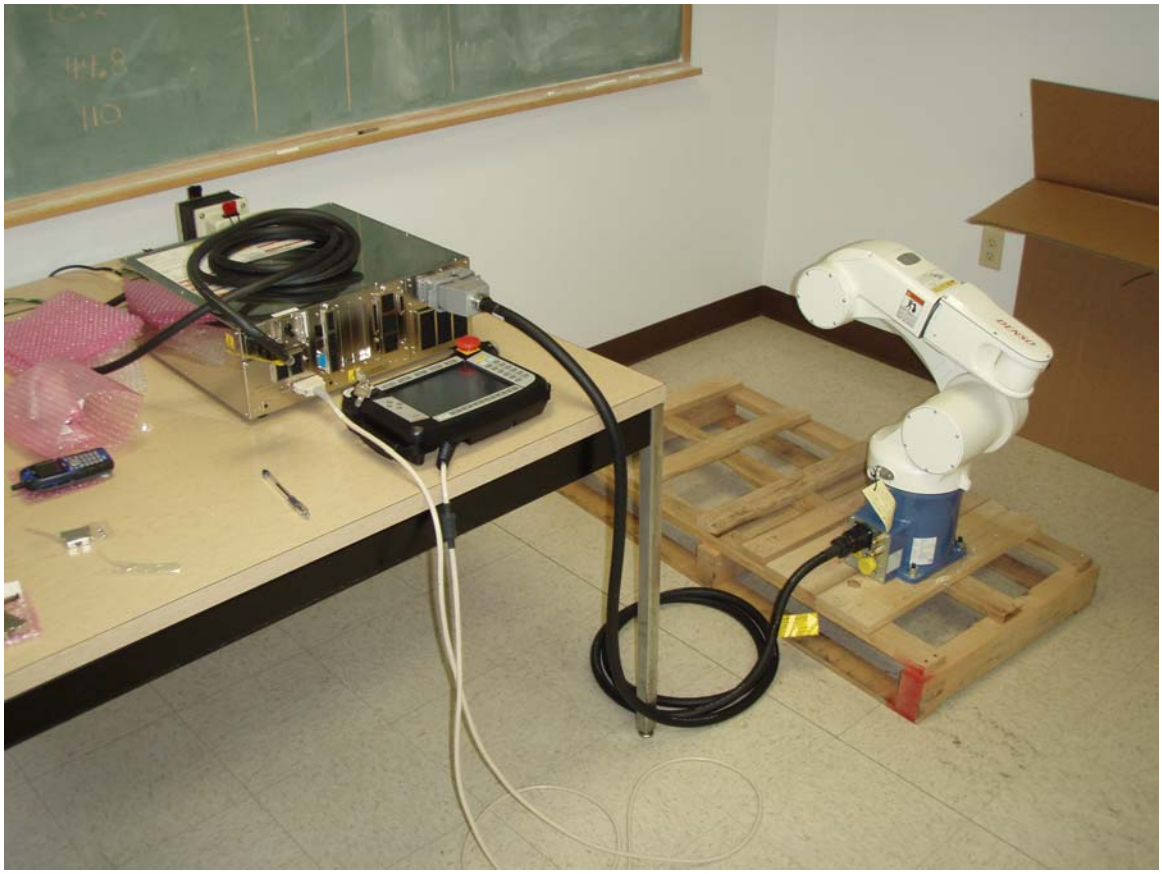

Figure 16 –Denso robot calibration.
This image shows the Denso robot being unpackaged and initially calibrated while still attached to the shipping skid in Fish Labs.

Phase 2 – Work Cell Design

Most automation systems are built into work cells for the purpose of enclosing the machinery and protecting users from entering the work envelope of the equipment. The work cell also provides the rigid structure that the equipment is mounted to. For this project for the author planned two work cells. The first work cell was used for prototyping system possibilities as well as research and development. After the final design was solidified a second and final work cell was built for routine embryo pick and place.

The first work cell was constructed inexpensively from lumber that allowed disassembly and transport because the areas that research was taking place in were under extensive renovation. The tool plate was made from a solid core fire door that provided enough rigidity and weight to mount the robot and other system components to.

Rigidity of the tool plate is an important factor in work cell design because relative motion of the base of the robot to other components in the system can cause major problems such as collisions and degraded poisoning accuracy. The work cell that was used for process research and development did not include protection to users as pending funds were not yet available. While not an ideal setup, this development phase posed inherently low risk of injury to the author (sole operator) as robotic arm speed never exceeded 5 cm/sec. Figure 17 - Work cell shows the work cell and tool plate during construction.

Figure 17 - Work cell tool plate.
This image shows the work cell including the solid core fire door that is being utilized as a tool plate shortly after being manufactured.

Phase 3 – Machine Vision

Once basic control over the robot was mastered, the next major step was integrating the Cognex machine vision cameras and the robot. They both have the ability to communicate over a number of mediums including Ethernet and RS-232. Ethernet communication was chosen to be the main form of communication within the work cell because it allowed the most flexible configuration.

Once the communications between the robot and machine vision cameras were established the physical setup of the machine vision system began. The most common and simplest way to set up a machine vision camera is to have it in a fixed location above the work area, so it never changes perspective in relation to the work plane. This setup necessitates a structure that can hold the machine vision camera above the work area which is then difficult to move around for prototyping purposes, and care must be taken to not have a collision between the robot and vision structure. An alternative design is to mount the machine vision camera to the robot itself as shown in Figure 18 - First setup with vision. This also allows changing the system to a new job with minimal reconfiguring. Mounting the machine vision camera onto the robot does come at a cost; the software is much more complicated because the camera is not in a fixed location. Instead of just mapping the coordinate system of the robot on the camera, a relative coordinate system must be made, and then scaled and shifted to the precise current position of the camera which, must have code to convert back and forth through the three different reference planes.

The system works by first moving the camera to a location over the work area. The robot then sends a command to the camera to take a picture; this command also has the current position of the camera included in it. The camera then takes an image and processes it. When the processing is complete, the camera computes the pixel location of the item; this is the location in the camera's relative work reference plane. The camera then converts the location in the pixel reference plane to a position in millimeters from the center of the CCD. This position is added to the position of the camera to convert it to the robot's reference plane.

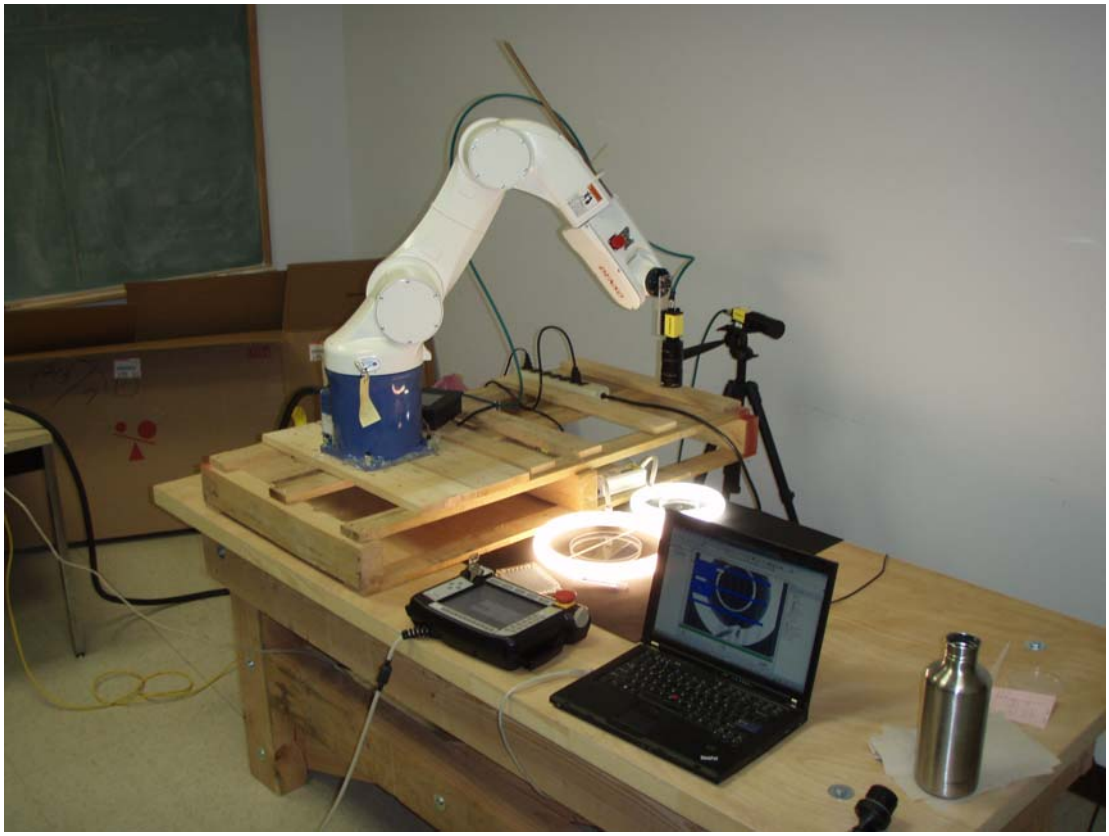

Figure 18 - First setup with vision.
This image shows the Denso robot still attached to the shipping skid sitting on the work cell tool plate while some initial vision testing is taking place.

The largest challenge to the entire project was generating enough contrast between the translucent embryos and the background.
Figure 19 illustrates the progression of increasing contrast with different lighting/backlight setups. Utilizing a backlight ended up washing out the image, rendering the embryos invisible. Utilizing ambient lighting with a white background was also unsuccessful as the shadows cast by the embryos were dispersed and not detectable. Placing a black background behind the embryos generated a promising image that allowed the machine vision camera to detect the embryos. While this setup of ambient light with a black background functioned well, it had major problems as ambient lighting conditions changed and with shadows or glare from the surrounding environment. To counter changes in the ambient lighting, a ring light was used to increase localized light on the embryos; this additional side lighting minimized lighting

changes from the environment as well as significantly boosting the contrast of the embryos.

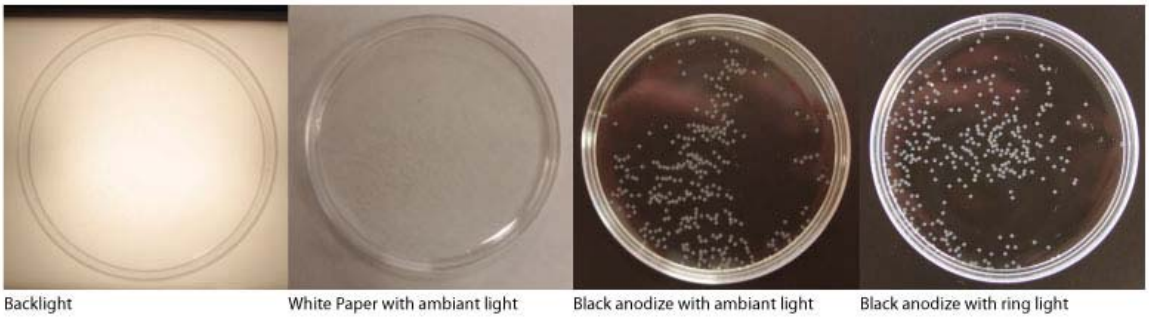

Figure 19 - Embryo contrast examples.
This image compilation illustrates the differences that lighting and background can make to the embryo contrast.

Phase 4 – End Effector

The end effector is the term for the device that is attached to the end of the robotic arm. For this project the end effector consists of three main components. The first component is the metal bracket that is attached to the robot. The micropipette is then attached to this bracket. While the machine vision camera also attaches to the end effector bracket, it is truly a system on its own and is not addressed in this section.

The end effector bracket was designed to allow for minimal interference with the robot's range of motion. It has two "wings" on either side as illustrated in the solid model shown in Figure 20. These wings allow for the micropipette and the machine vision camera to be mounted to the end effector bracket. The camera bolts directly to the bracket utilizing the camera's mounting base plate. The micropipette has a special bracket that allows the glass tube to be clamped in place using two set screws. The bracket can be swapped out to allow for different diameters of glass tube to be utilized. The actual end effector bracket with micropipette and machine vision camera is illustrated in Figure 21.

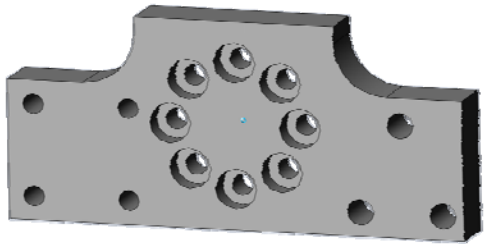

Figure 20 - Drawing of end effector bracket
Solid works model of the bracket that mounts the machine vision camera (right) and micropipette holder (left) to the robotic arm (center)

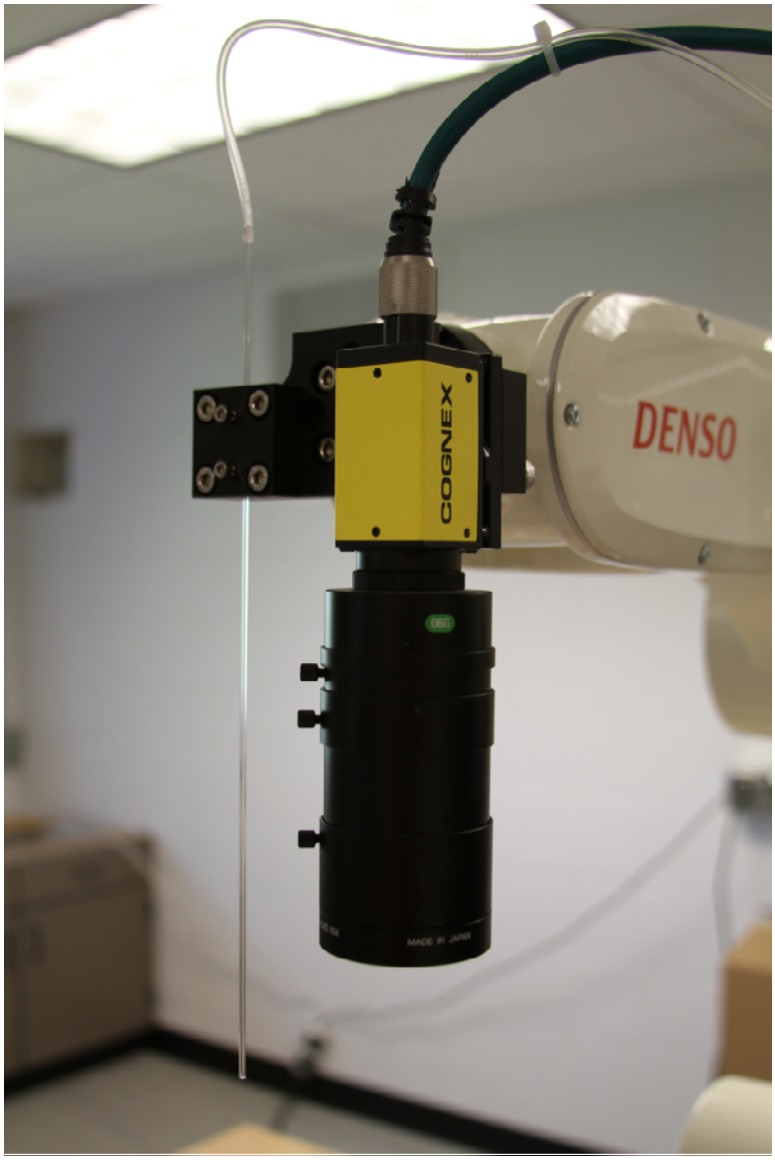

Figure 21 - End effector mounted on the robot arm.
End effector with micropipette on the left and machine vision camera on the right.

The glass micropipette tip proved more difficult than expected to produce. The tip needs to be a precise and polished or the embryos do not seal to the glass and can fall off. Once the glass has been cut, it is heated with a handheld torch to produce a rounded tip that has a constricted entrance. This provides more surface area for the embryo to rest against, minimizing the stresses exerted. The constricted entrance also reduces the number of embryos that are sucked into the tip causing it to plug.

Phase 5 – Final System Integration

Final system integration brought together the components developed in the prior four implementation phases; the robot, the work cell, the machine vision system, and the end effector.

The first step of this process was to mount the robot to the tool plate. Two large steel plates were utilized to help prevent keyholing of the fire door that was used as the tool plate; the forces generated by the robot during repeated accelerations can cause the thru holes to distort and allow the robot base to shift in relation to the tool plate.

The end effector bracket was mounted to the robot with both the micropipette and machine vision camera in place. Initially there was some concern with mounting the syringe pump to the robot, so for the first portion of testing it was left on the tool plate. This allowed it to function, but resulted in a longer tube connecting it to the micropipette which reduced its precision and occasionally became pinched or otherwise obstructed. Later, the syringe pump was mounted to the robotic arm and its function greatly improved.

Once the key components were assembled, several prototype system configurations were tested using various levels of complexity. Figure 22 shows the work cell during some of the initial trials of microinjection. In this illustration the syringe pump was not yet mounted on the robot arm and is sitting on the tool plate.

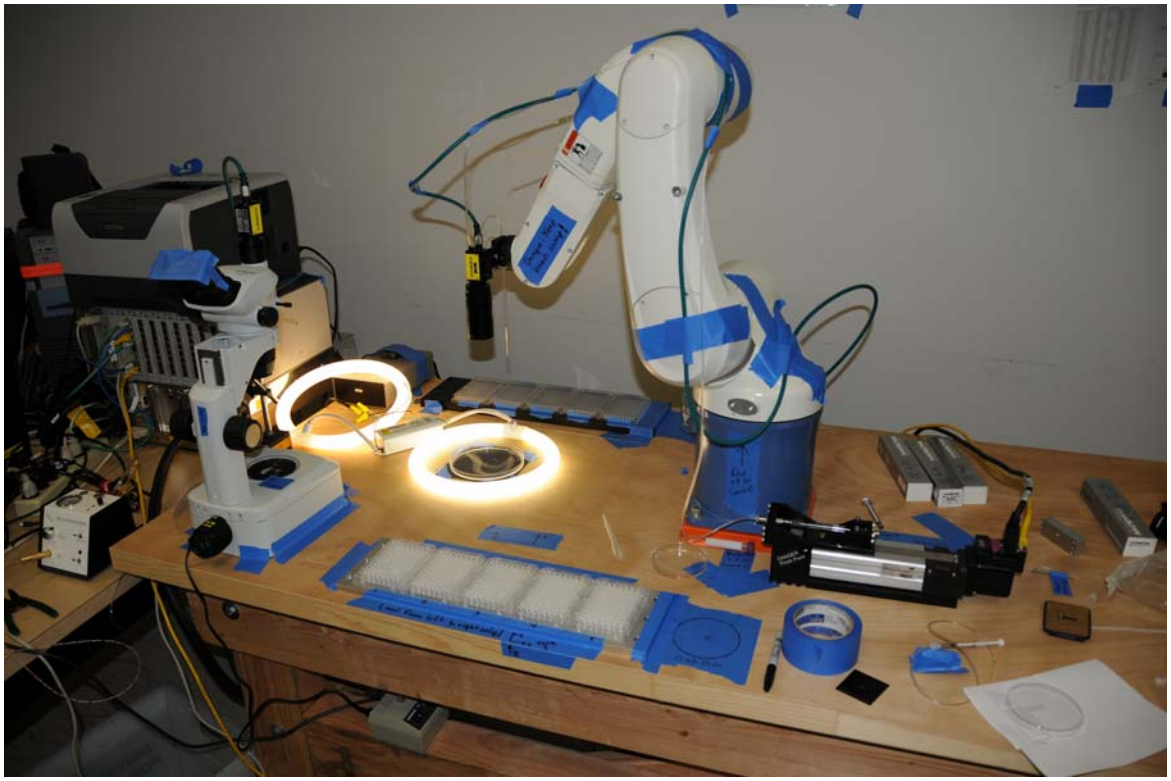

Figure 22 – Typical work cell setup for testing

Later on during testing the author focused on the simple pick and place of zebrafish embryos with no close-up inspection such as that shown in Figure 23. This system uses the robot mounted camera to locate embryos in the bulk solution tray and pick them up and place them into the 96 well plate. There is no close up inspection or ability for injection. This is the first system that was produced and it will be incorporated into the Tier-1 high throughput screening.

Figure 23 - Simple pick and place configuration

System Testing and Results

System testing was carried out by using the system to place embryos into 96-well plates. During this process, the desired performance parameters were evaluated to ensure that they meet the design requirements. The table below shows the results of the testing for each of the seven key requirements:

Requirement	Target	Acceptable	Achieved	Result
Picking up embryos	100%	95%	99.7%	Success
Putting embryos into 96 well plates without damage	100%	95%	99.5%	Success
Moving embryos with as little embryo solution (buffer) as possible	0uL	50uL	10uL	Success
Detecting embryos with visible necrosis	100%	95%	97%	Success
Visual inspection of embryos	100%	90%	100%	Success
Injection of embryos	100%	95%	40%	Failure
Precision dispensing of fluid	1uL	10uL	.1uL	Success

The results from testing were evaluated after the process was tuned to run efficiently. There were several nuances to the system that had to be standardized in order to achieve these results. With the system properly set up and operated, these results were consistently reproduced prior to the machine being disassembled and moved.

During testing it was demonstrated that the system could successfully pick up embryos 99.7% of the time. Only one embryo out of 4 plates was not picked up successfully. This indicates that the system in totality functions. It is able to locate an embryo, determine its location in the robot's coordinate system and successfully move to that location and pick up the embryo. The rate of success for the system picking up embryos exceeded the acceptable standard and was considered a success.

There was no indication of damage to any of the embryos that were placed into the wells during testing. There were only two different situations observed that led to embryo damage. The first and most problematic one was embryos sticking to the end effector. They would be picked up normally but then fail to drop when the system tried to eject them. This mainly occurred when the system was run after sitting for a period; the cause is believed to be a dry end effector. The solution to this problem was moving 10-20 embryos to waste while removing any that became stuck. A more permanent solution is to implement a process to "wet" the end effector at the beginning of every run. The second mode of failure was only observed on embryos 36 hpf and older. The chorion would break allowing the embryo to be drawn up into the pipette. This damage was anticipated because at 36hpf embryos are on the verge of hatching out. Embryos at 36 hpf were only handled for testing purposes and are clearly not suitable for pneumatic handling. Sticking of embryos <36hpf occurred with a near constant frequency and was not significant enough to degrade performance below the acceptable level allowing this requirement to be successfully met.

Measuring the amount of buffer that was transferred with the embryos was a difficult process. The amount transferred was significantly less than the amount that is transferred when manually pipetting embryos. Determining the amount of buffer moved with the embryo was done by visually comparing the amount of water in a well moved by the system with a known amount pipetted into a neighboring well. Through this process it was determined that the system could repeatedly move embryos with less than 10uL

of buffer. If an embryo was missed during the pickup, significantly more buffer would be picked up and deposited into the well, but this well would be considered a failure because of the missing embryo. The system successfully met this requirement as it was able to move significantly less buffer than the acceptable maximum.

Necrotic embryos were detected by their opacity difference from living embryos. The system was able to detect 100% of obviously necrotic embryos but failed to detect embryos that were in early stage necrosis with minimal opacity changes. If most of the embryo lacked signs of necrosis, the system could fail to detect it. This partial stage of necrosis only lasts a short period of time and the robot was able to detect enough of them to successfully meet the requirement.

Visual inspection of the embryos was preformed with a camera mounted on a dissection scope. It allowed the system to determine if an embryo was present on the tip of the pipette. With the resolution afforded by the dissection scope, the system was able to detect the presence or absence of embryos with 100% success. The high rate of success meets the target for this requirement.

Injection of embryos at first appeared to be a great success. Only two axes were monitored and controlled, and the system was able to consistently achieve proper positioning on those axes. The third axis was unmonitored and uncontrolled and this led to the low success rate. Embryo movement inside the chorion caused the needle to miss its target. This will be easily improved with the addition of a third camera and control of the third axis, but it was determined that for this project efforts should be applied elsewhere. This is the only requirement that was not successfully met.

The system could consistently dispense less than 100 nanoliters which was used as minimum volume achieved. Testing at 10uL and 100uL indicated that the system could repeatedly dispense a volume of 25 nanoliters. The precision achieved in dispensing successfully meet the requirement.

Chapter 6 – Conclusions

Key Findings

- Zebrafish eggs are able to be picked up and manipulated with a robotic system.
- When properly setup, a high level of reliability can be obtained negating the need for vision inspection of the work being performed.
- A process thought too “subjective” for automation is possible with new machine vision technology.
- Basic analysis of physical characteristics is possible during automated pick and place.

Key Implications

- Enable automated high throughput nanomaterials screening for biological consequences of exposure.
- Assessment of nanomaterial safety that precedes product development.
- The ability to have large on-demand capacity without idle costs.
- Increase productivity of research facilities and labs.
- Pharmaceutical drug discovery/development.

Key Contributions

- Technology to manipulate zebrafish eggs robotically.
- Basic machine vision analysis of zebrafish embryos.
- Reduced manual manipulation of samples.
  Skilled labor task are shifted from tedium to data analysis, interpretation and reporting.

Chapter 8 – Future Work

During this project much was accomplished, but there are still several functions that need to be added to this system to make it more robust and able to handle all of the situations requiring zebrafish embryos. Beyond this machine there are other areas in the high throughput screening that could be improved by automation.

Future work on this machine

The main feature that this machine still needs is the ability to handle dechorionated embryos. Some of the research carried out with zebrafish requires embryos that lack their protective shell. Currently the system can only pick up chorionated embryos because of the size and type of pipette that is used. Since dechorinated embryos are incredibly fragile, they cannot be picked up using suction to hold them on the end of the pipette; they must be drawn inside the pipette in the liquid column to avoid damage. This method may result in picking up more than one at a time but can probably be avoided with optimal embryo density and dispersal in the source dish. A pipette tip and methodology to handle dechorinated embryos is under development.

Another handling aspect the author developed is injection of chorinated embryos. This has been tested on the current system and functioned. Several improvements are needed for a fully functional system to be developed, but the fundamental operations have been refined and function successfully.

Future Manipulation Systems

Laser Amputation

One high impact area of research involving zebrafish is limb regeneration. Zebrafish can regenerate their fins and organs after injury. The Tanguay lab has pioneered the caudal fin regeneration model in zebrafish as a powerful platform for understanding how and why zebrafish can do this, but humans cannot. The fin regeneration studies seek to screen natural compound libraries containing thousands of compounds for leads that promote or inhibit regeneration. These leads are compounds that necessarily target the active cellular regeneration machinery and may target the largely inactive regeneration machinery that humans are thought to possess.

The regenerative work requires the amputation of the caudal tail. In the current low throughput approach amputation is a time consuming manual process. Higher throughput with manual amputation is impractical. But a rapid automated approach is likely possible with new technology in laser micro-dissection that cuts tissue with little damage to the surrounding area. A system incorporating this laser technology with machine vision to identify and amputate tails will be a powerful tool to finally discover drug leads for regenerative medicine.

Adult Injections

Targeted adult injections are another process that is time consuming to set up and requires a number of people. This is where an adult zebrafish is restrained in a fixture and nanomaterial is injected into a specific area of the fish, such as the abdomen or brain. Utilizing an automated system to restrain the adult zebrafish while keeping it submerged in water, and machine vision to identify the proper area for the injections, and control the injection, would reduce the amount of time laboratory technicians need to spend on this tedious task. Consistent injections with automation would also reduce the variance in the data and likely lower detection limits.

Future Behavioral Monitoring Systems

Another area critical to developmental toxicology research and thus critical to develop automated platforms for is behavior. Currently the high content imager is the only automated solution that can examine zebrafish embryos in a high throughput way but it is incapable of dealing with a moving subject and thus, cannot address behavior. To date, behavior is the most sensitive developmental toxicology endpoint, with measurable changes in simple features like tail bending frequency and swimming propensity being impacted by exposures far below the concentration needed to elicit a classical morphology change.

Behavioral testing is practical on adult as well as embryonic zebrafish. Commercially available behavior platforms for embryonic zebrafish do not exist. For larval stage zebrafish, the only commercial system (ViewPoint Life Sciences, Inc., Montreal, CAN) is poorly constructed and is controlled by software that is highly unstable. For adult behavior essentially no commercially available platforms exist. A robust and reliable system that is easily tied into current data streams in needed for each of the three desired behavioral endpoints, embryonic, larval, and adult.

Embryonic Behavior

At the embryonic stage the only observable movement in zebrafish is the bending of their tail. This bending is arrhythmic and can be easily observed using machine vision. Exposure to low concentrations of several neurotransmitter receptor binding compounds in the lab indicates that tail bending frequency can be dramatically modulated up or down soon after addition of the chemical to the embryo water. This was tested using a test setup as a proof of concept, shown in Figure 24 - Embryonic Behavior Test. Nicotine, a substance already known to cause a dramatic increase in tail bend frequency, was introduced to the water holding 24 hpf embryos. The author's design monitored and a detected the tail bend frequency increase and, significant for throughput needs, was able to perform this task on multiple embryos per well of a 96 well plate. Figure 25 shows the results of the nicotine test. Two wells were monitored, the red line is the control which had no nicotine added, and the blue line (with the two spikes) had low doses of nicotine added at each of the spikes. In both cases there is a distinct increase in activity that slowly drops back down to normal levels or below.

The index of activity is calculated by monitoring all the pixels that make up a well image and determining each pixel's grayscale as a 16 bit value (between 0 and 255). The next image is taken and again each pixel is compared to the same one in the previous image and the differences in the 16 bit grayscale values are summed across the entire well. In theory, if there is no motion, then both images will be exactly the same and the relative index will be zero. If the embryo bends its tail, the two images will be slightly different and a positive relative motion index will be greater than zero. In order to reduce the signal to noise ratio, 10 embryos are placed into each well to help average out their motion. The software for this application was provided with the Cognex camera used and with minor coding proved to be user friendly, stable control of the design. Moreover, the data output was in a form readily importable by MS Excel, for analysis.

Figure 24 - Embryonic Behavior Test
Illustrates an experimental setup to test the ability to detect and track embryonic movement with a machine vision camera.

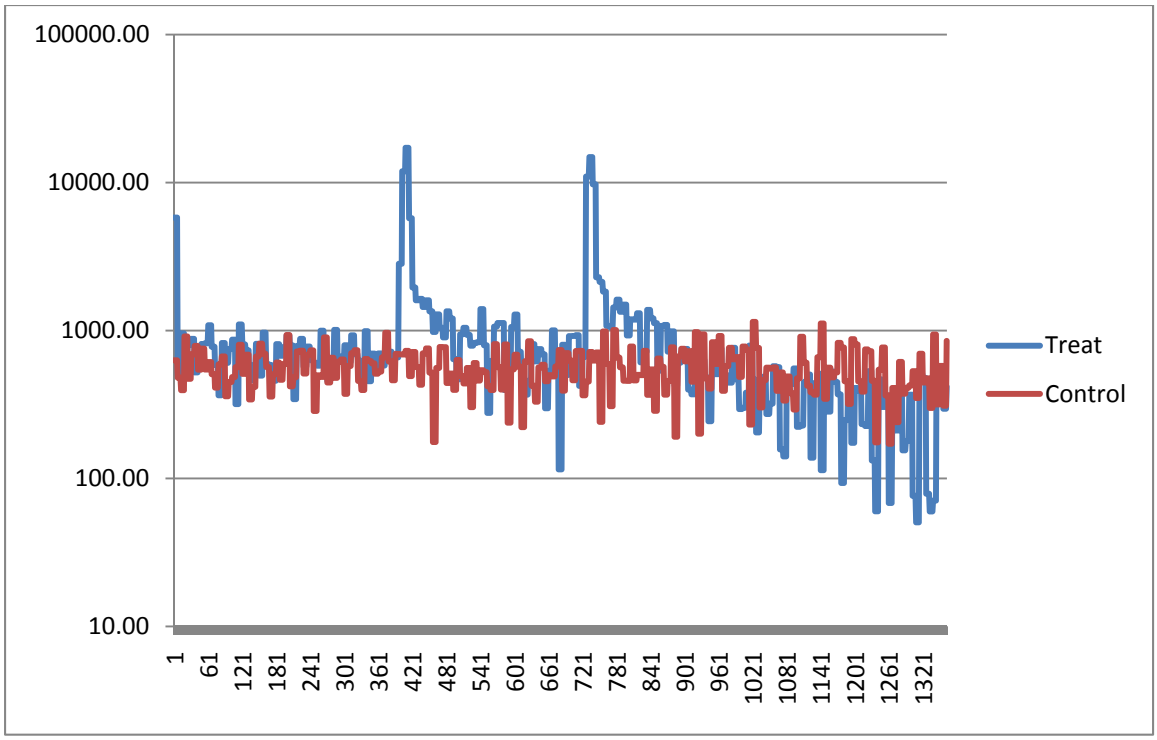

Figure 25 - Embryonic Behavior Test with Nicotine
Chart produced by embryonic behavior system showing two spikes in movement associated with the application of doses of nicotine.

Larval Behavior

Larval behavior characterization involves tracking the movements of a single larval zebrafish inside the well of a 96 well plate. The zebrafish are backlit with an IR light that allows the camera to see them as the visible light is turned on and off for a stimulus. The fish are tracked as they move around the well and comparisons between the amount of motion when they are in the visible light, and when they are in the dark can be compared.

The author's solution to the challenge used a double telecentric lens allowing for the creation of a relatively compact unit that has no moving parts to keep aligned, and provides an extremely large objective to enable the machine vision system to capture undistorted images of each well of an entire 96 well plate with minimal IR illumination..

Adult Behavior

Adult behavior characterization is the least understood of the three behavioral areas. The ability to track zebrafish in three dimensions while swimming in a tank gives many opportunities for new characterization. In addition adult zebrafish display aggression behavior, shoaling behavior and cognitive learning and memory of simple tasks involving food reward and/or non-harming punishment. Images of predator fish or other zebrafish can be displayed on LCD monitors alongside the tank to assess aggression/stress or schoaling behavior, respectively. Learning and memory can be tested with mazes and different stimuli including audible, visual, and electrical.

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