



InFocus

Center of Comparative Medicine & Pathology
Research Animal Resource Center
Laboratory of Comparative Pathology



Zoonotic Hazards Associated with Zebrafish (Danio rerio)

There are a variety of diseases that can be acquired from aquatic animals such as zebrafish (*Danio rerio*) used in biomedical research. Zoonotic diseases are diseases of animals that are transmissible to humans. The overall prevalence of disease transmission from fish to people is low¹. Most zoonoses associated with fish result from ingesting fish contaminated with various bacteria and parasites. Laboratory fish associated zoonoses have only been associated with bacteria. The bacteria can be transmitted by accidental ingestion of contaminated water due to splashing, secondary infections of open wounds by exposure to contaminated water, and direct contact with infected fish¹. In the majority of cases these infections are observed in and pose the greatest risk to immunosuppressed individuals as these zoonotic bacteria are opportunistic. The following are the most common laboratory acquired bacterial infections associated with fish:

Mycobacteria marinum and *M. fortuitum* are ubiquitous, non-motile,

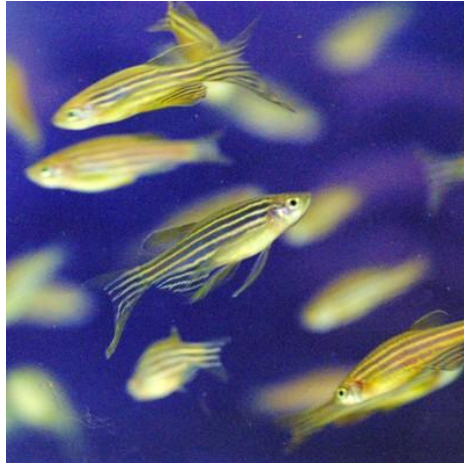
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Avoiding Death as an Endpoint: A Necessary Refinement

Animal use regulations emphasize the need to minimize pain and distress in animals used in biomedical research. While such regulations clearly state that animal welfare must be addressed, they do not prescribe how to meet the expectation. The Three R's (replacement, reduction, and refinement) proposed by Russell and Burch (1959) provide a strategy, which has been widely accepted, for meeting the goal of minimizing pain and distress. They define refinement as "a decrease in the incidence of severity of inhumane procedures applied to those animals which have to be used." Choosing humane endpoints for scientific experiments demonstrates refinement in that it minimizes or eliminates the amount of time animals experience pain and/or distress. Well defined endpoints ensure that experiments are terminated before the animals suffer or that animals are euthanized should they experience pain and/or distress.

In general allowing animals to succumb without intervention in many experimental situations, is considered inhumane and

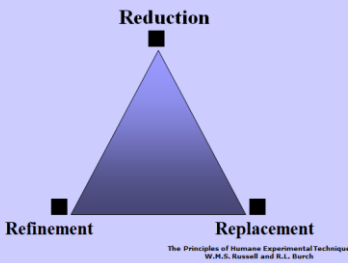
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The increasing use of the zebrafish, *Danio rerio*, in biomedical research presents new challenges welcomed by the CCMP.

Image credit: http://www.digs-bb.de/digs-bb/images/science/Zf_tank.jpg/view

The Three R's Russell and Burch, 1959



Reduce, Replace and Refine- Tools for meeting the goal of minimizing pain and distress in animals used in research testing and teaching.

Human LCMV infection: Why it is critical to test cell lines/stocks and biologics for contamination

In April, a patient with aseptic meningitis was diagnosed lymphocytic choriomeningitis virus (LCMV) infection. The patient worked at an Indiana rodent breeding facility raising rodents for the pet trade. After an extensive investigation by the CDC, thirteen other employees at the site tested positive for LCMV, nine of which showed clinical illness. All recovered without complications. Mice in the facility tested positive for LCMV.

This outbreak shows striking similarities to a 1992 LCMV outbreak at a cancer research institute in Texas where 8 employees were identified as having been infected with LCMV. The source was nude mice inoculated with a tumor cell line unknowingly infected with the virus. All employees infected had been working with the cell line and nude mice.

LCMV may be identified in the laboratory as an accidental finding as highlighted

above. However, the virus is also an important research tool. LCMV has been used extensively as a model of persistent viral infection to investigate various aspects of antiviral immunity, immune suppression and tissue rejection mechanisms. This model was used by Peter Doherty and Rolf Zinkernagel who received the Nobel Prize in Medicine for their work (using this model) demonstrating how the immune system recognizes virus infected cells.

At MSKCC/WCMC we have implemented policies to prevent inadvertent introduction of LCMV and other zoonotic agents into our colonies. Most importantly, cell lines/stocks and biologics of rodent origin or that have been passaged through rodents must be tested for infectious agent contamination prior to in vivo use. Additionally, all work with cell lines/stocks of human origin must be

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Inside:

- ✓ New Faces in the CCMP
- ✓ Progress in Flood Control and Prevention

Dr. Michelle Lepherd achieves dual certification from the American College of Veterinary Pathologists

In September, Dr. Michelle Lepherd received certification in Clinical Pathology from the American College of Veterinary Pathologists (ACVP). Dr. Lepherd was certified in Anatomic Pathology in 2011, and she is now among a small number of ACVP members who have achieved certification in both disciplines. After obtaining her BVSc and PhD degrees from the University of Sydney, Dr. Lepherd completed her Anatomic Pathology residency at Cornell University's College of Veterinary Medicine, and her Fellowship in Comparative and Genomic Pathology within our department.

Michelle will join the staff of the Laboratory of Comparative Pathology (LCP) in November as a Comparative Pathologist. Her expertise and certification in Clinical Pathology will allow the LCP to expand services in hematology, clinical chemistry, and cytopathology.



Dr. Michelle Lepherd certified in both Clinical Pathology and Anatomic Pathology joins the staff of the Laboratory of Comparative Pathology



CCMP welcomes Punita Koustubhan, to a newly created position- Aquatics Systems and Services Manager



Investigator Communication Initiative Program

As of July 1, 2012, RARC began the implementation of the *Investigator Communication Initiative Program*. The purpose of this program is to promote communication, understanding, and cooperation between investigative staff working in the vivarium and various RARC sections which will contribute to an even higher quality of animal care.

Every other month, RARC will contact select laboratories to coordinate a meeting with specific laboratory members and representatives from various RARC sections to discuss any issues of concern as well as training opportunities, veterinary care, and husbandry. To facilitate the discussion, a survey form will be sent out to the laboratory to be filled out by all members prior to the meeting. Participation in the program is optional but will be encouraged.

RARC looks forward to this new initiative to improve the care being provided and promote cooperation and communication within the College.

Image credit: mysuccessfullife.co.uk

Punita Koustubhan, Manager, Aquatics Systems and Services

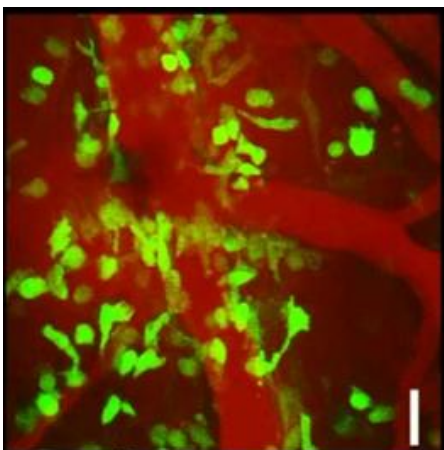
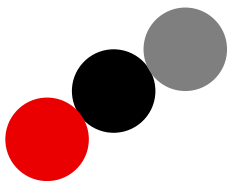
The Center of Comparative Medicine and Pathology (CCMP) is pleased to welcome Punita Koustubhan, as Aquatics Systems and Services Manager, a position developed to provide specialized oversight for all aquatic species housed at both MSKCC and WCMC. Punita received her Bachelor of Science in Biology with a minor in Marine Science from Richard Stockton College of New Jersey. She has over 10 years of experience managing zebrafish and anuran facilities at Tufts University and received an Advanced Certification in Laboratory Animal Science from the Forsyth Institute, an affiliate of Harvard Medical School and the Harvard School of Dental Medicine. Most recently, Punita served as the Center Laboratory Manager for the Tufts University Center for Regenerative & Developmental Biology, where she designed and supervised the construction of a 700-gallon, fully automated *Xenopus laevis* housing system. Punita has in-depth experience designing aquatic systems and managing aquaculture facilities in an academic research setting and has authored several research publications in her areas of expertise. Please welcome Punita to the CCMP. Her office is located in the CCMP office suite in the Zuckerman Research Center (Z 921) and she can be contacted via email at koustubp@mskcc.org or 646-888-3478.

LCMV infection, Cont. from pg. 1

conducted at Animal Biosafety Level-2 (ABSL 2) using personal protective equipment and biological safety cabinets. Additional information regarding testing can be found on our website: (<http://mskweb5.mskcc.org/intranet/html/83970.cfm> or http://intranet.med.cornell.edu/research/rarc/ani_ser/map_rap_test.html?name1=Biologicals+Screening&type1=2Active)

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- Notes from the Field: Lymphocytic Choriomeningitis Virus Infections in Employees of a Rodent Breeding Facility – Indiana, May-June 2012. *Weekly August 17, 2012* 1(32);622-623
- Dykewicz CA, Dato VM, Fisher-Hoch FP, Howarth MV, Perez-Orozco GI, Ostroff SM, Gary H, Schonberger LB, McCormick JB. Lymphocytic Choriomeningitis Outbreak Associated With Nude Mice in a Research Institute. *JAMA*. 1992;267(10):1349-1353



Perivascular monocytes and macrophages mediate vascular leakage in LCMV meningitis

Image credit: <http://saturn.med.nyu.edu/research/mp/dustinlab/jkim-homepage/movies.html>

Zebrafish Zoonoses, Cont. from pg. 1

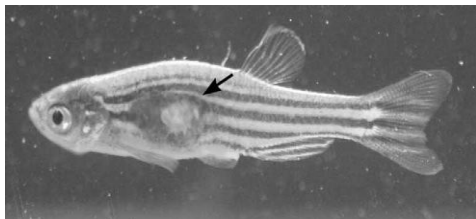
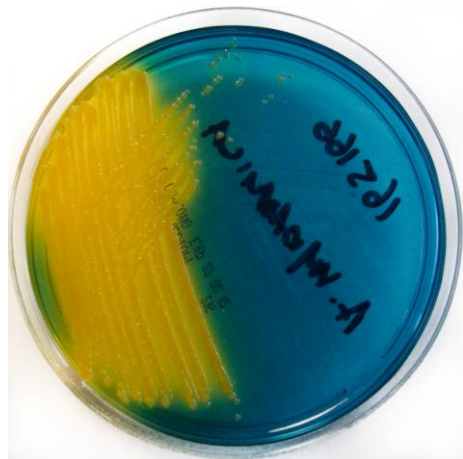


Image 1. Image of a zebrafish with skin ulceration due to *M. marinum*⁴. Lesion is identified by an arrow.



Image 2. *M. marinum* nodules and hand inflammation of the proximal joints of the digits⁵.



Aeromonas hydrophila on TCBS Agar.jpg
Image credit:
http://commons.wikimedia.org/wiki/File:Aeromonas_hydrophila_on_TCBS_Agar.jp

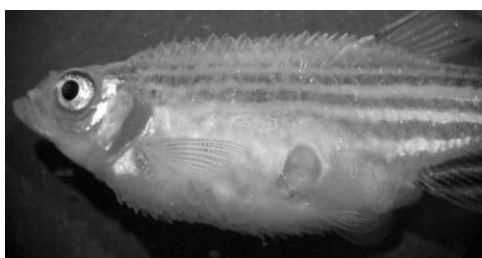


Image 2. Photo of a zebrafish with severe edema, raised scales and distended abdomen⁴.

acid-fast rods that are pathogens of both fish and humans. In humans they cause a disease commonly known as fish handler's disease or fish tank granuloma¹. Zebrafish infected with this bacterium may have ulcerative lesions on their bodies. Typically, humans are infected via contamination of a skin laceration or abrasion with fish system water or direct contact with contaminated fish. This disease appears most commonly as a localized, hard, granulomatous nodule that develops at the site of infection². The granulomas may appear 6-8 weeks after exposure to the bacterium^{1,2}. They can also appear as non-healing ulcers¹. The infection can also spread to local lymph nodes and may become generalized in severely immunocompromised people². Treatment for individuals with only skin lesions is generally a combination of clarithromycin combined with a variety of other antibiotics such as ethambutol and rifampin⁵. The prognosis is good but treatment may require up to 3-4 months of therapy. In immunocompromised patients with deep tissue invasion, surgical debridement of the granulomas combined with combination antibiotic treatment is usually necessary to be curative⁵.

Aeromonas hydrophila, *A. caviae*, *A. sobria* and *A. schubertii* are facultative anaerobic, gram-negative rods that have been identified in a number of freshwater and marine fish, including zebrafish². In zebrafish, it can cause ulcerative skin lesions, raised scales, abdominal distention, exophthalmia and septicemia leading to death^{1,3}. The most commonly isolated species is *A. hydrophila*. It is a normal commensal found in the gastrointestinal tract of fish. The two most common clinical syndromes in infected people are gastroenteritis (nausea, vomiting and diarrhea) as well as localized wound infections^{1,2}. This infection can be debilitating in immunocompromised individuals as it can cause a necrotizing fasciitis^{1,2}. When identified early prognosis is good as the bacteria is susceptible to monotherapy with fluoroquinolones, aminoglycosides and chloramphenicol. It is resistant to first generation cephalosporins and penicillin⁶.

Streptococcus iniae is a ubiquitous, gram-positive, non-motile beta-hemolytic cocci that has been most commonly identified in freshwater fish housed in recirculating systems¹. Zebrafish are

extremely susceptible to this organism and they can become infected secondary to overcrowding and poor water quality¹. There have been reports of people becoming infected with this bacterium after injuring themselves while working with contaminated water or infected fish. The most common clinical sign is cellulitis; however, there have also been reports of infected individuals developing endocarditis and meningitis¹.

Pseudomonas aeruginosa, another ubiquitous gram-negative bacterium, is found in water and soil¹. It is a common cause of hospital-acquired infections, especially in immunocompromised patients¹. There have been no reports of natural infections of this bacterium in zebrafish, but in some cases they are used as animal models to study the pathogenesis of the disease in people, therefore there is a potential they could harbor the bacterium¹.

Erysipelothrix rhusiopathiae is a gram-positive rod that is ubiquitous in soil and water. It does not appear to be pathogenic in fish; however, fish can harbor this bacterium on their skin or in their mucus coat³. Humans become infected when handling fish, as the bacteria enters through a skin wound. Several syndromes have been described in infected individuals³. The first is a localized skin infection that occurs at the site of the wound. The second is a diffuse cutaneous form when it has progressed to the surrounding tissues. A systemic form affecting the heart and heart valves, which may lead to death, has also been described.

There are also many bacteria in the Enterobacteriaceae family that have the potential to cause zoonotic disease³. These include *Edwardsiella spp*, *Escherichia spp*, *Salmonella spp*. and *Klebsiella spp*. These are all gram-negative, facultative anaerobic rods that are associated with water or fish. While *Edwardsiella ictaluri* and *E. tarda* are major pathogens of many fish, they have not been reported in zebrafish. People become infected via contamination of wounds on the hands during fish handling. Clinical signs associated with infection include necrotic skin lesions and gastroenteritis³.

Other bacteria that are typically spread via incidental ingestion of contaminated water, or improperly prepared fish include *Plesiomonas shigelloides*, *Pseudomonas fluorescens*, *Staphylococcus spp*, *Nocardia spp*, *Cryptosporidium spp*,

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Zebrafish Zoonoses, cont. from pg. 3

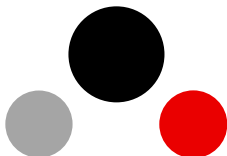
Safety First!

- Follow proper **QUARANTINE** procedures on incoming fish
- **GLOVE UP** at all times
- **WASH UP** thoroughly when finished
- **FIRST AID** to cuts/abrasions immediately
- **Minimize SPLASHING!**

An ounce of prevention...
Disease transmission from fish to people is low. Keep it that way by adhering to basic safety guidelines.



Image credit: <http://kimandjason.com/blog/2008-03-26/27-ways-to-stomp-out-spring-stress.html>



Clostridium spp, *Campylobacter spp* and *Vibrio spp*¹⁻³. These bacteria typically cause gastrointestinal disease in people who become infected.

There are a variety of ways to prevent infection from these pathogens. The first is to prevent entry of these pathogens to the aquatic systems that house the fish by respecting appropriate quarantine procedures, performing bacterial culture on incoming fish, and only introducing bleached embryos onto a clean system³. Gloves should be worn at all times when working with zebrafish, as even with appropriate quarantine measures, some bacteria are ubiquitous in the environment and may infect humans through skin abrasions³. After working with fish, hands and forearms should be thoroughly washed¹. Minor cuts or abrasions should immediately be rinsed and cleaned and protected from exposure to aquarium water and fish¹. It is also important to minimize splashing when working with fish and water to prevent inadvertent ingestion of water.

While the overall incidence of disease transmission from zebrafish to humans is low, it is important to remain vigilant and understand the risks associated with working in an aquatic environment. Finally, while this article has focused on zoonotic diseases that may be acquired from zebrafish, other hazards exist in the aquatic environment that can pose a risk

to personnel. These include slips, trips, electrocution, and exposure to chemicals used in experiments. Always follow appropriate safety guidelines in order to ensure your safety when working in the fish rooms or procedure areas.

References:

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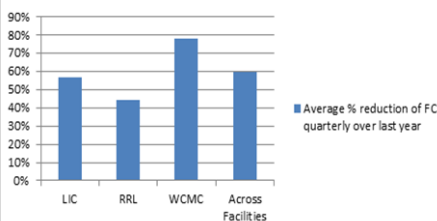
Progress in Flooded Cage Control and Prevention

You may have or know of someone in your lab that has experienced a flooded rodent cage. This can be detrimental to the cage occupants (animal health and to your experiments). RARC has been working diligently to identify the principal causes of the flooded cages in our vivaria, meticulously recording the circumstances and other pertinent information available when a flooded cage is found. We have been investigating various types of thermoplastics used in bottle production, including bottle lifespan and manufacturing practices, to establish what bottle type to use to replace existing bottles as they age and as our facilities expand. Additionally, we have been keeping detailed records to determine other variables that may cause bottles to fail and cages to flood. We have been replacing bottles greater than 6 years old. In addition to and in conjunction with our continuing

investigation, we have been testing all water bottles overnight after filling them to ensure they do not leak. All bottles are now inverted and held at least 12 hours to ensure they remain full the following day prior to placement in the cage. Any bottles that are found empty after this test are inspected and removed from circulation.

We would like to share our results to date. Over the first three quarters of 2012 during which bottles were replaced and inverted, we have seen a dramatic reduction (60% average across all facilities; see table) in the quantity of flooded cages as compared with the last three quarters of 2011, before these changes were implemented. Although we still see flooded cages, the reduction has been significant. Importantly, we continue to make further changes to reduce flooding with the aim to reduce flooding to rates that are negligible.

Average % reduction of FC quarterly over last year



Results to date over the first three quarters of 2012 indicating a dramatic reduction in the incidence of flooded caging.



Avoiding Death as an Endpoint, Cont. from pg. 1

Aids in determining humane endpoints

- Search the literature for alternative endpoints
- Investigate the disease course seen in humans
- Create a clinical or morbidity scoring system
- Conduct a pilot study with fewer animals
- Identify biomarkers predictive of morbidity.
- Provide additional staff training to recognize deviations from normal species behavior

Early recognition of the late stages of disease may allow investigators to avoid the use of death as an endpoint and to collect data relevant to meet the scientific objectives of the study.

does not meet the regulatory expectation that the 3 Rs be implemented.

One question that arises is how does one conduct scientific experiments to study disease conditions that often result in death? The simplest approach is to preemptively euthanize animals that are about to die or that are in the process of dying. The latter is referred to as the moribund condition. A clear scientific advantage of euthanizing animals before death is that it allows for collection of data or samples that may be impossible to collect, or be of little value, if acquired after death. The period leading up to the moribund condition may be associated with pain, stress or distress. While samples and data can be collected from moribund animals, moribundity in itself can cause physiologic changes that may render collected data inaccurate. Therefore the expectation is that animals should be euthanized before becoming moribund.

Since a moribund animal has potentially experienced significant pain and distress, there is a need to identify those animals at an earlier stage. There are several approaches that can be considered. When using well-characterized models, alternative endpoints may be described in the literature. Selecting these endpoints is beneficial as it allows for comparison between studies and the endpoints have often been validated as predictive of outcome. This avoids the worrisome possibility of missing data or misinterpreting results which may occur by ending an experiment too soon. If there is minimal or no literature available on the specific animal model, investigating the human disease may provide useful information on the expected disease course that can be used to select the endpoint.

Another approach is to create a clinical or morbidity scoring system, by developing a metric using a combination of factors to assign a score to each animal based on various parameters (behavioral, physiologic, biochemical, etc). When the morbidity score has deviated by a pre-defined amount from normal, the animal is euthanized before it becomes moribund.

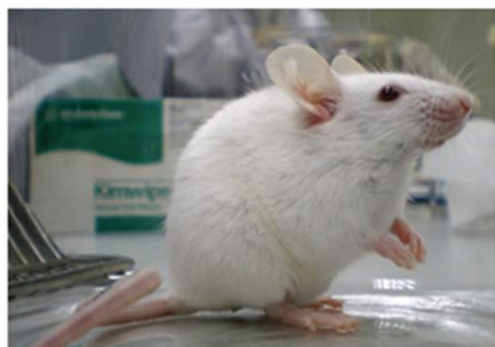
When using new or uncharacterized models, one should consider conducting a preliminary or pilot study with a reduced number of animals. A pilot study that includes frequent observations provides insight into the course of disease and allows for identification of potential endpoints, which if used, will limit the number of animals that suffer pain and

distress. It also allows for more accurate planning when conducting studies with larger cohorts. Frequent observation is important in pilot studies, or any study when the course of disease is unpredictable, as with newly-created genetically modified animals. The use of humane endpoints, in general, requires frequent animal observation.

Close observation may allow for the identification of biomarkers. Biomarkers, in this context, are parameters that accurately predict morbidity or death before the actual state is reached. Biomarkers, like other endpoints, vary depending on the type of study and its scientific goals. For example, a biomarker for a study of tumor development may be the presence of a measurable tumor, while a study evaluating treatment efficacy may require measuring a serum biochemical indices over time. Biomarkers have been developed for a variety of cancer and infectious disease models. For instance, hypothermia, or a drop in body temperature about 4-6°C has been proven highly predictive of death in several rodent infectious disease models (Trammell and Toth 2011). Hind limb paralysis is commonly used as an endpoint in some rodent models, such as leptomenigeal tumors in rats (Janczewski 1998) and amyotrophic lateral sclerosis (Solomon 2011). Additional biomarkers used in cancer models include tumor doubling time or size as well as the presence of specific clinical signs or paraneoplastic syndromes. Generic “biomarkers” that are applicable to a wide array of studies include weight loss, inability to ambulate, decreased responsiveness to external stimuli (handling, conspecifics, etc), physical appearance, loss of the righting reflex, body condition scoring, and reduced food or water consumption.

It is ethically and scientifically imperative to avoid the use of death as an endpoint, unless absolutely necessary to meet the scientific objectives of the study. Fortunately alternatives have been identified. It is important to recognize that alternative humane endpoints are not universal; they must be tailored to each model. The use of alternatives to “death as an endpoint” often allows the scientific objectives of the protocol to be met while addressing welfare concerns. In general, the use of humane endpoints requires frequent observation as well as staff training and

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ILAR JOURNAL

The ILAR Journal, the quarterly, peer-reviewed publication of the Institute for Laboratory Animal Research, provides thoughtful and timely information for all who use, care for, and oversee the use of laboratory animals.

Refer to the following online publication for more information:

Humane Endpoints for Animals Used in Biomedical Research and Testing

@ http://dels-old.nas.edu/ilar_n/ilarjournal/41_2/

Image credit: <http://dels.nas.edu/ilar>

Advances in the Care of Laboratory Zebrafish

The use of zebrafish (*Danio rerio*) in biomedical research has increased exponentially over the past twenty years. However, information regarding the care and use of these animals in the research setting has lagged behind. Unlike rodent animal models, basic standards for nutrition, housing, husbandry procedures and welfare are not well-established. This has the potential to lead to differing results from research studies conducted at different institutions maintaining and utilizing zebrafish. To address this need, Gadsden State Community College (GSCC) and the University of Alabama at Birmingham (UAB) entered into a partnership to develop a standard training curriculum for individuals involved in the care of laboratory zebrafish. In June '12, 18 world experts in zebrafish husbandry, aquaculture, laboratory management, veterinary medicine and regulatory compliance convened to develop the curriculum which will include on-line training presentation and a hands-on workshop. Details of the program will be presented at the national meeting of the American Association for Laboratory Animal Science (November 4-8, 2012). The on-line course is expected to launch in early 2013 with the first technical workshop to begin in the summer of 2013. Dr. Christine Lieggi, RARC's Associate Director, was one of the invited experts involved in the development of this program and she can be contacted for additional information at lieggi@mskcc.org, or chl2019@med.cornell.edu.

Avoiding Death as an Endpoint, Cont. from pg. 5

experience to recognize deviations from the animal's normal behavior. CCMP staff are available for consultation on pain and distress recognition as well as to assist in the selection of specific endpoints for your model.

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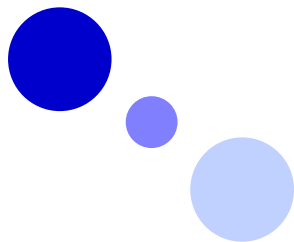
Gadsden State hosts zebrafish workshop



Participants in the Zebrafish Husbandry Education Curriculum Development Workshop use a net to harvest fish, Monday in Gadsden. The workshop is being held at Gadsden State's Aquaculture Department.

MARC GOLDEN | GADSDEN TIMES

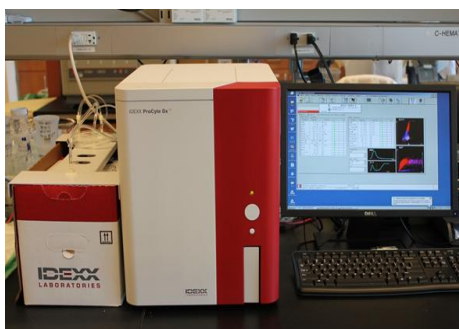
Article credit:
<http://www.gadsdentimes.com/article/20120604/NEWS/120609932>



LCP Acquires New Hematology Analyzer Requiring Extremely Small Blood Volumes

The Laboratory of Comparative Pathology (LCP), has acquired a new multi-species hematology analyzer which requires extremely small sample volumes making it ideal for analysis in small laboratory animals such as mice. The instrument has species specific protocols for the analysis of blood from a variety of laboratory species used at MSKCC and WCMC including mice, rats, rabbits, pigs and non-human primates. Requiring a sample volume as small as 30 microliters the instrument is ideally suited for repetitive hematologic analyses of blood from individual mice. A comprehensive CBC is produced including a 5 part WBC

differential, standard RBC measurements and platelet indices and it also provides a reticulocyte count separating the reticulocyte population into percentages of low, medium and high fluorescence intensity as a measure of erythropoietic response. Results can be provided to users in an Excel spreadsheet for subsequent analysis or printed as a report (pdf format) that includes color histograms of the WBC differential and reticulocyte populations along with single parameter histograms of the RBC and platelet populations. The LCP will be establishing species, strain, sex and age specific normal ranges to facilitate data interpretation.



New technology allows for safer sampling and comprehensive hematologic testing of small laboratory animals.