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Best practices for germ-free derivation and gnotobiotic zebrafish husbandry

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Abstract

All animals are ecosystems with resident microbial communities, referred to as microbiota, which play profound roles in host development, physiology, and evolution. Enabled by new DNA sequencing technologies, there is a burgeoning interest in animal–microbiota interactions, but dissecting the specific impacts of microbes on their hosts is experimentally challenging. Gnotobiology, the study of biological systems in which all members are known, enables precise experimental analysis of the necessity and sufficiency of microbes in animal biology by deriving animals germ-free (GF) and inoculating them with defined microbial lineages. Mammalian host models have long dominated gnotobiology, but we have recently adapted gnotobiotic approaches to the zebrafish (*Danio rerio*), an important aquatic model. Zebrafish offer several experimental attributes that enable rapid, large-scale gnotobiotic experimentation with high replication rates and exquisite optical resolution. Here we describe detailed protocols for three procedures that form the foundation of zebrafish gnotobiology: derivation of GF embryos, microbial association of GF animals, and long-term, GF husbandry. Our aim is to provide sufficient guidance in zebrafish gnotobiotic methodology to expand and enrich this exciting field of research.

INTRODUCTION

HOW TO USE THIS CHAPTER

The goal of this chapter is to make information available to researchers interested in embarking on gnotobiotic experiments in zebrafish. We provide detailed protocols and discussion of three types of gnotobiotic procedures: derivation of germ-free (GF) embryos, microbial association of GF animals, and long-term, GF husbandry. We describe specific techniques and provide illustrative figures, with special attention to strategies for avoiding contamination—the bane of gnotobiotic research. Stand-alone tables that collate reagents and protocols for each procedure follow the text. We hope this chapter serves as a useful guide for researchers interested in pursuing zebrafish gnotobiology.

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IMPORTANCE OF GNOTOBIOLOGY RESEARCH

The body surfaces of all animals are inhabited by resident microbial communities, referred to as microbiota, which play profound roles in host development, physiology, and evolution (McFall-Ngai et al., 2013). Advances in DNA sequencing technologies have enabled descriptions of animal-associated microbiota composition in exquisite detail. Indeed, large descriptive studies have identified numerous correlations between microbiota composition and host health (Human Microbiome Project Consortium, 2012). However, manipulative experiments are needed to test these relationships and to elucidate mechanisms by which microbiota affect their hosts. The term “gnotobiology” (*gnos*, known; *bios*, life) was coined to describe the study of animals raised in the absence of microorganisms or in the presence of known microbial strains or communities (Reyniers et al., 1949). In this way, much as a geneticist performs genetic loss- and gain-of-function experiments, gnotobiologists remove resident micro-biota to test necessity and add back one or more microbial strains to test sufficiency.

Model organism choice for gnotobiotic studies is motivated by host processes under investigation, extent of experimental replication necessary to achieve statistical power, and feasibility to maintain sterility or defined microbial conditions. Gnotobiology has a rich history across many organisms, especially in laboratory mice. Our groups have established zebrafish as a gnotobiotic model (Bates et al., 2006; Rawls, Samuel, & Gordon, 2004) to take advantage of this animal’s outstanding experimental attributes, including rapid external development, optical transparency, large brood size, and ease of housing (Grunwald & Eisen, 2002). These properties enable vertebrate gnotobiotic experimentation on a scale not possible with mammalian models.

We have used gnotobiotic zebrafish to study microbiota effects on the host as well as host effects on the microbiota. For example, we established that the resident microbiota are necessary for host intestinal epithelial maturation and turnover, lipid absorption, and immune homeostasis (Bates, Akerlund, Mittge, & Guillemin, 2007; Bates et al., 2006; Cheesman, Neal, Mittge, Seredick, & Guillemin, 2011; Kanther et al., 2011; Rawls et al., 2004; Rolig, Parthasarathy, Burns, Bohannon, & Guillemin, 2015; Semova et al., 2012). In some cases, generic bacterial products, such as lipopolysaccharide are sufficient to mediate microbiota effects, whereas in other cases microbial signals are more specific to particular bacterial lineages (Bates et al., 2006; Rawls, Mahowald, Ley, & Gordon, 2006). We have also demonstrated that the host modulates bacterial dynamics and competition within the intestine (Jemielita et al., 2014; Rawls, Mahowald, Goodman, Trent, & Gordon, 2007; Taormina et al., 2012; Wiles et al., 2016) and that gnoto-biotic zebrafish can be used to identify determinants of microbial colonization, including diet (Semova et al., 2012; Stephens et al., 2015; Wong et al., 2015). Gnotobiotic zebrafish have also been used to study colonization resistance against pathogens (Rendueles et al., 2012). Future research directions are discussed in the Section 2 at the end of this chapter. We hope that this chapter inspires research endeavors that will enrich our collective understanding of animals as microbial ecosystems.

HISTORICAL PERSPECTIVE ON GNOTOBIOLOGY

The first concept of studying GF life is credited to correspondence in 1885 between Louis Pasteur (1885) and Émile Duclaux (1885). Stimulated by Duclaux's efforts to germinate beans and peas in the absence of microbes, Pasteur expressed interest in rearing animals from birth devoid of microbes, predicting that animal life without microbes would be "impossible," hypothesizing that microbiota must be beneficial, or essential, to their animal hosts. Pasteur's famous prediction sparked theoretical debate and experimental tests of whether animal life was feasible in the absence of microbes. The first successful production of GF animals, in this case guinea pigs, was reported 10 years later by Nuttall and Thierfelder (Nuttall, 1896). This initial success was followed by reports of GF derivation of chickens, goats, and a range of other mammals, birds, and amphibians (Baker, Ferguson, & TenBroeck, 1942; Gordon, 1960). These early studies showed that deriving animals GF was relatively straightforward, but resulted in variable longevity and health status. The difficulties in maintaining healthy GF animals prolonged debate over the feasibility of GF animal life. In hindsight, this variability can be largely attributed to insufficient diets. Michel Cohendy (1912), working with chickens provided the first convincing demonstration that GF animals could be reared for prolonged periods with generally good health. These advances were supported by technological developments in GF gnotobiotic housing, first by Ersnt Küster (2013).

With these technical advances, the scientific inquiry of gnotobiology shifted from GF animal viability to the impact of microbes on animal life. These research questions motivated further developments in gnotobiotic isolator designs and materials (Gordon, 1960; Reyniers et al., 1949; Reyniers, 1959). Although current gnotobiotic isolators look similar to those of the mid-20th century, demands of the evolving gnotobiotic research field have motivated new innovations, such as methods for scalable gnotobiotics (Laukens, Brinkman, Raes, De Vos, & Vandenabeele, 2016).

The greatest challenge in gnotobiotic husbandry has been to develop sterile diets that support growth and survival of GF animals. A diet must meet nutritional requirements to effectively support animal growth and survival. Gnotobiotic research has revealed that the absence of microbiota and their metabolic capabilities significantly augments animal nutritional needs that must be met by diets even after undergoing sterilization, which may significantly reduce their nutritive content. This challenge became apparent early in gnotobiotic animal research, as variable growth and survival in early GF animal studies was attributed in part to inadequate diets. Advances in formulation and sterilization of GF diets, most notably discovery of vitamins (Semba, 2012) enabled gnotobiology to move forward. However, nutritional and biochemical contributions of microbiota to host biology continue to be discovered (Krishnan, Alden, & Lee, 2015), suggesting that even well-established standard diets for gnotobiotic mice and other animals may still have "insufficiencies" that could underlie observed differences between GF and colonized animals. This underscores the need to continue to study microbial contributions to host nutrition and to develop new diets that may more completely meet nutritional needs of GF animals.

Within this rich history of gnotobiotic research, fish models have played a small yet increasingly important role. The first fish species to be derived GF was platyfish

(*Xiphophorus maculatus*) and survival on sterilized diets was achieved for several weeks after yolk resorption (Baker et al., 1942). Successful GF derivation was subsequently reported for many species including tilapia (Shaw, 1957), threespine stickleback (Milligan-Myhre et al., 2016), multiple salmonid (Lesel & Lesel, 1976; Trust, 1974) and Poeciliidae species (Lesel, 1979), sheepshead minnow (Battalora, Ellender, & Martin, 1985), turbot (Munro, Barbour, & Birkbeck, 1995), Atlantic halibut (Verner-Jeffreys, Shields, & Birkbeck, 2003), sea bass (Dierckens et al., 2009), Atlantic cod (Forberg, Arukwe, & Vadstein, 2011), and threespine stickleback (Milligan-Myhre et al., 2016). These pioneering efforts confirmed that fish can survive under GF conditions for a limited time, but did not define nutritional requirements, explore GF phenotypes, or succeed in growing GF fish to reproductive maturity. As zebrafish emerged as a powerful model for biological and biomedical research, it was a natural choice to be developed as a gnotobiotic model. As described earlier, gnotobiotic zebrafish has become a powerful platform for mechanistic studies of associations between animals and their resident microbiota.

CONTROLS FOR GNOTOBIOTIC EXPERIMENTS

Model organisms with defined wild-type strains have enabled experimental reproducibility between laboratories across the world. However, all animals are microbial ecosystems, and individuals differ in the composition and function of their resident microbial consortia, making experimental reproducibility more elusive (Stappenbeck & Virgin, 2016). Indeed, mouse immunologists and infectious disease researchers have long known that mice purchased from different vendors exhibit different immune phenotypes and susceptibilities to pathogens. More recently, many examples of mouse genetic models of chronic gastrointestinal disorders such as inflammation have been plagued with the problem that robust phenotypes observed in one laboratory are not reproducible when mice are shipped to another laboratory. Even within one experiment in a single laboratory we documented enormous inter-individual variability in microbiota composition in a group of identically reared sibling zebrafish (Stephens et al., 2016). How then, should an investigator approach the challenge of defining a colonized control group against which to compare GF or other gnotobiotic groups?

The gnotobiology field has struggled with this question for decades. One approach is to use a standardized, simplified microbiota as a surrogate for a natural community. In the mid-1960s Russell W. Schaedler developed a defined inoculum for GF mice, “Schaedler Flora,” composed of specific mouse bacterial isolates (Dewhirst et al., 1999). This inoculum was further developed by Roger Orcutt in 1978 to an eight strain combination currently used by mouse researchers and referred to as the “Altered Schaedler Flora.” Standardized reference microbiota have been further championed by Andrew Macpherson and Kathy McCoy (2015), who coined the term “isobiotic,” analogous to “isogenic,” as a standard for making experimental comparisons to infer microbiota function between individuals and across experiments. Macpherson and McCoy recognize the major logistical hurdles to performing experiments in the same microbiological “background,” for example, requiring shipment of gnotobiotic animals around the world, but they assert that this level of replication is important for fields such as developmental immunology.

Another approach, articulated by Thad Stappenbeck and Skip Virgin (2016), is to accept but document the enormous variability in microbiomes within and between experiments. These authors stress the importance of characterizing the functional capacities of microbiota by performing microbiota transplantations as a strategy to determine whether a given phenotype in an experimental animal is explained more dominantly by its host genotype or its microbial genotype. Although this approach is labor intensive and costly, it highlights important considerations for the field.

We struggle with these questions as well. In practice, we favor focusing research on microbiota impacts that are robust across control groups and readily observed when comparing GF animals to a range of colonized animals. We typically use one of two control groups: conventional (CV) animals reared without any microbial manipulations, and conventionalized or ex-GF (CVZ or XGF) animals derived GF and then inoculated with a natural source of zebrafish-associated microbes. We still debate optimal timing of conventionalization [typically 3–4 days post fertilization (dpf) when zebrafish normally hatch from their protective chorions] and the source of inoculum (typically water from the parental crossing or “system water” that circulates in a conventional fish facility). We have experimented with zebrafish equivalent of a Schaedler Flora, referred to as a “Zebrafish-Associated Community” (“ZAC”), assembled based on knowledge of the major zebrafish microbiota constituents and composed of bacteria we isolated from healthy zebrafish and for which we have genome sequences (Stephens et al., 2016). However, we are reluctant to employ such a microbial community as our sole comparator, because we still have so much to learn about zebrafish microbiota. Indeed, our research has highlighted impacts of minor microbiota members (Rolig et al., 2015) as well as the importance of stochastic processes in zebrafish microbiota assembly (Burns et al., 2016) that call into question the feasibility of generating cohorts of zebrafish with stereotyped, defined microbial communities. We encourage other investigators to consider the best controls for their experiments, depending upon the questions they wish to address.

1. EXPERIMENTAL PROCEDURES

In this section, we describe the latest methodology for successful gnotobiotic zebra-fish research.

1.1 IMPORTANT CONSIDERATIONS FOR GNOTOBIOTIC RESEARCH

The starting point for all gnotobiotic zebrafish research is derivation of GF embryos. The methodology for this procedure is straightforward, but successful derivation and maintenance of sterility require careful planning, repeated practice, and attention to detail. We discuss both requirements that should be considered before attempting to derive embryos GF and some challenges and common pitfalls to successful GF derivation and husbandry.

1.1.1 Physical space considerations for optimal sterility—Space considerations for GF derivation, bacterial associations, and long-term GF husbandry should include cleanliness, airflow, sink locations, human traffic patterns, and biosafety level status. All GF experimentation requires walls, floors, and work surfaces that can be easily cleaned and maintained. Surfaces should be bleach- and ethanol tolerant. Filtered air is preferable and

friable ceilings and unfiltered duct-work should be avoided to prevent airborne contamination. For all gnotobiotic husbandry, we recommend working with GF and conventional zebrafish in separate, dedicated spaces with separate tools and supplies. Ideally, personnel complete their daily tasks with GF animals prior to performing any procedures with conventionally reared zebrafish or bacterial cultures to minimize potential for cross-contamination. Dedicated GF space should be uncluttered, storing only sterile materials and reagents critical for experiments, and sources of contamination such as human traffic, dust from room surfaces, and aerosolized materials, should be minimized. All items should be cleaned with 70% ethanol prior to room entry. We discourage sink use in the dedicated GF room due to splashing, aerosolization, and inadvertent spread of contaminants.

Because each of the experimental procedures we discuss—GF derivation, microbial association, and long-term husbandry—has unique requirements, if possible it is desirable to have separate rooms dedicated to each function, preferably within close proximity to the main fish facility and other necessary equipment such as incubators, autoclaves, and centrifuges.

Space for GF derivation of zebrafish embryos: GF derivation of embryos requires space devoted solely to that purpose. It should have a dedicated biosafety cabinet (BSC), a door that closes, and counter-space. Air pressure should be positive to the surrounding room or corridor to minimize external airborne contamination. Human traffic in the space should be minimal while the derivation procedure is in progress.

Space for microbial association of GF zebrafish: Microbial association of GF embryos and larvae should be performed in rooms that are easily decontaminated. Rooms should be Biosafety Level 2 (BSL-2) approved when using microorganisms that could be pathogenic to humans or fish or that carry antibiotic-resistance genes. Ideally, microbial association should be performed in a separate BSC from that used for GF derivations, or on a laboratory benchtop with careful sterile technique. Procedures for housing microbial-association experiments in temperature-controlled rooms and incubators should be clearly established, including proper signage for other users of the space.

Space for long-term husbandry of GF zebrafish: Long-term husbandry of GF and gnotobiotic zebrafish is an area of active exploration and our existing protocols for this procedure have not been as well tested as our short-term larval husbandry. When practical, long-term husbandry of GF zebrafish should occur in a dedicated room maintained at 28°C with a 14 h light/10 h dark photoperiod. Because cumulative risk of contamination increases with experiment duration, long-term GF husbandry requires a room designed to minimize contaminant introduction. In our experience, humans are the most common vectors for contamination, thus room access should be limited to trained investigators who always wear gloves and dedicated personal protective equipment (PPE) such as laboratory coats or smocks. Schedules should be arranged so that personnel only work in the dedicated GF room before interacting with conventionally reared fish or bacterial cultures.

1.1.2 Equipment considerations for preventing contamination—Gnotobiotic experimentation relies heavily on several types of equipment. It is vital that investigators

receive standardized training on equipment functionality, use, care, testing, and certification requirements. In the following we discuss two pieces of equipment that are critical for GF zebrafish experimentation:

Biosafety cabinet: Zebrafish embryos are most reliably derived GF in a BSC. To use a BSC properly, investigators must understand how airflow protects both sample and user from contamination, how to work within a BSC environment to preserve sterility, the major routes of contamination, what can and cannot be achieved by UV illumination, how to clean the BSC, and how to maintain BSC certification. All supplies in the BSC should be cleaned with 70% ethanol and investigators should wear a laboratory coat, disposable smock, or sterile sleeves. The BSC should be supplied with dedicated pipettes, filter pipette tips, pipet aids, tube racks, and pens. It is a misconception that UV light is the best method for disinfection of the BSC and its contents. Surface disinfection of the BSC, its contents, and users' gloved hands and arms with 70% ethanol is the most reliable strategy to minimize contamination. BSCs work well for GF derivation of zebrafish embryos if used appropriately, but do not prevent contamination if used inappropriately.

Autoclave: Autoclaves play a crucial role in preparing reagents and supplies for gnotobiotic research. Therefore, it is imperative that users have a basic understanding of their instrument and know how to reliably test whether it is functioning properly. Sterilization requires the entire volume of liquid to reach temperatures effective at eliminating the full spectrum of microbes present. Temperature can be monitored with autoclave probes and manufactured spore tests can reveal autoclave sterilization effectiveness.

1.1.3 Importance of healthy zebrafish stocks for germ-free experiments—The complexity of GF experiments demands special attention to animal husbandry that maximizes outcomes of lengthy experiments. Zebrafish facilities benefit from regularly sampling sentinel fish and a variety of facility locations for known pathogens as well as adopting practices that limit pathogen transmission (Mason et al., 2016). To ensure starting GF experiments with large clutches of robust embryos, we recommend using young (<12 months old), healthy adult zebrafish with special attention to maintaining as much background diversity as possible, screening each generation for developmental anomalies, and selecting for fecundity. For experiments with mutant or transgenic lines, optimizing parental stock genotypes maximizes experimental success. For experiments requiring individuals carrying a homozygous recessive mutation, one strategy to minimize genetic variation between the mutant individuals and the wild-type control siblings is to generate them from a mating of heterozygous parents. In experiments requiring large numbers of individuals, for which genotyping offspring would be prohibitive, breeding separate lines of homozygous mutant carriers and wild-type stocks may be required.

1.1.4 Training is critical for gnotobiology success—Standardized training is crucial for successful GF derivation of zebrafish embryos. Because personnel turnover is common in academia, it is critical to develop consistent training protocols. Training should include principles of zebrafish husbandry, sterile tissue culture technique, and equipment care and use. We recommend three training sessions: in session one trainees shadow the mentor, in

session two trainees work alongside the mentor, and in session three trainees perform procedures and the mentor critiques. The mentor then determines whether the trainee can perform procedures successfully without direct supervision. Because it is critical that all laboratory members understand the shared responsibility of maintaining sterility and preventing contamination, we recommend a “certification” so that everyone involved knows who has the appropriate skills and training to perform GF experiments.

1.2 BEST PRACTICES FOR GERM-FREE DERIVATION OF ZEBRAFISH EMBRYOS

Fig. 1 provides an overview of critical GF derivation procedures, Table 1 shows a sample time line, and Tables 2 and 3 list materials and specific procedures. Healthy adult zebrafish are placed in breeding cages with dividers separating males and females the afternoon or evening prior to spawning (Day –1). At the start of the light cycle the following morning (Day 0), the breeding insert is transferred to autoclaved water and adults allowed to spawn for no more than 60 min, to minimize exposure to microbes from the parents and to ensure a synchronized clutch of eggs for disinfection. Embryos are collected and chorions rinsed free of large particulates before transfer to a petri dish of filter-sterilized embryo medium containing antibiotics (ABEM) to reduce bacterial growth as embryos develop to the appropriate age for surface disinfection. Prior to surface sterilization, infertile and deformed embryos are removed, healthy embryos are sorted to a density of no more than 150–200 per dish, rinsed multiple times in fresh ABEM, and incubated for at least 5–6 h to maximize the antibiotic disinfection period. Between 50% epiboly and embryonic shield stage (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), embryos are surface disinfected with rinses in dilute polyvinylpyrrolidone-iodine (PVP-I) and bleach (sodium hypochlorite) and then incubated in sterile tissue culture flasks with vented lids. Flasks are checked daily for health and survival. If hatching has not occurred by 3 dpf, larvae are mechanically dechorionated by flicking the flask to knock embryos against the side of the container. Dechoriation prevents developmental deformities caused by physical constraints of the chorion.

Daily visual inspection of flask contents with phase contrast microscopy allows detection of adherent and motile bacteria and fungi, nonculturable microbes, and microbes present at very low density (Fig. 2). By 4 dpf, contamination can readily be detected by inspection of the flask using phase microscopy and culturing a sample on rich microbiological media such as tryptic soy (TSA), Luria broth (LB) agar, or Brain–Heart Infusion (BHI) at 28–30°C under aerobic and anaerobic conditions. We recommend practicing examining flasks containing fish tank water, conventionalized embryo medium (EM), and sterile EM, to learn the spectrum of organisms and debris within the experimental environment. Microorganisms within the water column are regularly shaped, dark, and may exhibit directional movement different from the Brownian motion or hydraulic flow of inert particles (Fig. 2A). This may include bacteria, mold, spores, and protozoans (Fig. 2A and B). Reflective, irregularly shaped objects are often debris such as pieces of chorions and microscopic plastic shavings present inside tissue culture flasks (Fig. 2C), and may occur even in GF flasks. Culturing samples from flasks aerobically and anaerobically on rich media such as TSA allows detection of culturable contaminants suspended in the water column. Plating also provides the ability to identify potential contaminants by colony morphology, colony polymerase chain reaction (PCR), and sequencing of small subunit ribosomal RNA genes. Tables 1–3

provide an overview of timing, supplies, and procedures for GF derivation of zebrafish embryos.

For GF experiments it is important to have a control group of zebrafish colonized by conventional microbiota. As described in the Introduction, these could be either CV or CVZ zebrafish, depending on the goals of the experiment.

1.3 MICROBIAL ASSOCIATION OF GERM-FREE ZEBRAFISH

Microbial colonization from the water column begins at about 3.5–4 dpf when the intestine is open at both the mouth and the anus. The density of fish influences bacterial colonization dynamics, so the density of fish per flask should be consistent, generally about 1 fish/mL. Prior to inoculation with microbes, sterile technique should be used to remove dead larvae, as they can be a source of nutrients for introduced microbes. We recommend inoculation at 4 dpf, to ensure all fish can be colonized, and because by this stage contaminated flasks can be easily detected and then (B) 20 μ M. removed from the experiment. Inoculum size should be determined empirically for each microbial species. For many bacteria, 10^4 – 10^6 colony-forming units (CFUs)/mL final concentration is sufficient to achieve intestinal colonization within 24 h at 1 fish/mL with minimal toxicity. These bacterial densities are selected to mimic the densities of culturable microbes observed in conventional aquaculture facilities. In the United States, experiments involving exposure of zebrafish to commensal microorganisms, especially those that could pose a health risk, may require Institutional Animal Care and Use Committee approval and should adhere to BSL-2 guidelines.

Fig. 3 describes methods for inoculating fish with commensal bacterial species that we have used for several bacterial isolates (e.g., Rolig et al., 2015). Table 4 describes the necessary materials and Table 5 provides the work flow and a sample calculation for a zebrafish isolate of an *Aeromonas* species. Optimal media and in vitro growth conditions vary for different bacterial species. Prior to mono-association with a single bacterial strain, or association with a consortium of bacterial strains, it is important to identify appropriate media and to optimize growth conditions to achieve an appropriate and reproducible bacterial density for colonization (Fig. 3A). Some bacterial strains will grow well in liquid culture directly from a frozen stock, whereas others may require streaking frozen stocks on appropriate agar plates, followed by 24 h incubation at 28–30°C before liquid culture inoculation with a single colony.

Once parameters for a particular inoculum are determined, prepare the culture for introduction into the GF flask as described in Table 5 and according to the specific experiment (Fig. 3B). First, flask sterility is verified by culturing on TSA (Fig. 3C). Inoculum should be introduced using sterile technique; actual bacterial density in the flask should be verified immediately following inoculation and again at the experimental end point.

1.4 LONG-TERM GERM-FREE ZEBRAFISH HUSBANDRY

Understanding the myriad ways resident microbes influence host development and physiology requires rearing zebrafish GF past early larval stages, with the ultimate goal of rearing them GF throughout their lifespan. The vast majority of gnotobiotic zebrafish

research to date has focused on larval stages, in part due to the challenges of long-term GF zebrafish husbandry methods. These challenges mirror those of running a miniature, specialized zebrafish facility, thus animal care and use regulations should inform all practices. Long-term gnotobiotic zebrafish husbandry requires consideration of animal housing, water quality and exchange, waste removal, nutritional value of food, live food culturing, work space requirements, standardized work flow, record keeping, supplies, expenses, and labor (Fig. 4).

Procedures for long-term GF husbandry are still evolving; Tables 6 and 7 show current materials and work flow. We have successfully raised GF zebrafish for up to 1 month, but in all cases they were significantly smaller than their counterparts reared in the main zebrafish facility on conventional diets. Our current methodology is extremely labor intensive, and must be carried out every day of the experiment, thus we recommend practicing with conventional zebrafish and establishing a personnel schedule for the duration of the experiment before embarking on long-term GF husbandry. It is also important to calculate the number of embryos and flasks that is needed based on: (1) experimental design (number of time points, biological replicates, internal controls for developmental stage and maturation); (2) anticipated survival rates (from previous GF experiments); (3) expected contamination rates (from practice sessions); and (4) expected genotype ratios (from parental genetics). We also rear at least 25 zebrafish from the cross in facility main housing, as a control for general embryo health. In our experience, 12 flasks each of CVZ and GF animals require three people working about 4–6 h per day, 7 days a week, including general maintenance. We discuss key parameters for long-term GF husbandry in the following sections.

1.4.1 Germ-free live food—There is limited knowledge of zebrafish stage-specific nutritional requirements (Watts, Powell, & D'Abramo, 2012), thus the single greatest challenge for long-term GF zebrafish husbandry is supplying adequate nutrition. Our best success has been with GF cultures of *Tetrahymena thermophila*, ciliates that are highly motile, elicit normal hunting behavior of zebrafish larvae, and help maintain flask water quality by consuming fecal matter and dead fish. To improve their nutritional content, we developed methods to culture *Tetrahymena* on sterile, emulsified, chicken egg yolk (Fig. 5), resulting in engorgement with numerous internal vacuoles compared to media-reared *Tetrahymena* (Fig. 6). In controlled feeding trials, zebrafish reared on egg yolk–cultured *Tetrahymena* had better survival and were larger than siblings reared on media-reared *Tetrahymena* (Melancon, Sichel, and Kelly, unpublished). Tables 8–10 provide the reagents and protocols for GF *Tetrahymena* culture propagation and egg yolk nutritional enhancement.

The most common route of GF experiment contamination is daily feedings, thus sterile technique and surveillance of GF *Tetrahymena* stocks are imperative. GF *Tetrahymena* cultures should be started at least a month in advance of any long-term GF experiment and if maintenance stocks become contaminated, new GF stocks should be ordered from the *Tetrahymena* Stock Center (<https://tetrahymena.vet.cornell.edu/>).

1.4.2 Media exchange, water quality, and surveillance—Another major challenge for long-term GF zebrafish husbandry is maintaining water quality. We typically change about 75% of the media daily, and use removed media to monitor water quality and contamination. The water quality of GF zebrafish co-cultured with GF *Tetrahymena* is relatively stable with low ammonia levels. More challenging has been maintenance of CV controls because of the tendency of the conventionalized flasks to develop microbial overgrowth resulting in poor zebrafish health. We are developing continuous flow cultures (see Section 2), which we hope will keep microbial growth under control during long-term culture.

1.4.3 Project management and record keeping—Just as running a zebrafish facility requires planning, allocation of responsibilities, and careful record keeping, so too does undertaking long-term GF zebrafish experiments. We recommend allocating responsibilities among participating personnel, including media and plate preparation, *Tetrahymena* maintenance cultures, and supply logistics. All participating personnel must be cross-trained so they can rotate through daily tasks that occur not only throughout the week, but also on weekends and holidays.

It is invaluable to keep detailed records during long-term GF experiments, enabling continual improvement of procedures, pinpointing possible contamination sources, and improving reproducibility. Developing a standardized method of daily note taking, with established checklists, facilitates efficient record keeping and communication among rotating staff. We use a separate checklist for each husbandry task and a standardized platform for recording data and notes. For each flask, we record flask plating results, visual inspection results, and numbers of sick and dead animals. In addition, we maintain a daily summary sheet that records media exchange volumes, feeding volumes, and ammonia levels.

2. PROSPECTUS

Zebrafish are a powerful model for studying diverse aspects of animal development, physiology, immunity, toxicology, and evolution. Gnotobiotic methods described here have begun to elucidate the diverse contributions microorganisms make to zebrafish biology. By providing detailed methods, we hope to stimulate broader adoption of gnotobiotic investigations in the zebrafish model and to gain new insights into host–microbiota relationships. Looking forward, we anticipate several important challenges that this research community will need to address.

2.1 ZEBRAFISH NUTRITION

Zebrafish husbandry has historically used a combination of live and artificial diets (Lawrence, 2007). Although live diets are highly palatable and widely used, their macro- and micronutrient content is often poorly defined or unknown, and can vary considerably across facilities. By contrast, artificial diets are manufactured with defined chemical compositions in mind. Despite the prevalence of zebrafish as a model, specific dietary requirements remain undefined (Watts et al., 2012). As a result, there are no uniform feeding practices, thus zebrafish research cannot take full advantage of chemically defined diets. Because the impact of diet composition and specific nutrients on zebrafish development and physiology

remains largely unexplored, we need to be aware of diet as a possible confounding factor across studies. The limitations of our current understanding of zebrafish nutrition will become more evident with the increased popularity of zebrafish as a model for physiological and pathophysiological processes where diet is a crucial environmental factor. Moreover, these limitations are amplified in GF husbandry, where in the absence of the nutritional contributions of microbes, missing nutrients need to be supplemented in the diet.

Development of GF diets has combined live and artificial diets. Initially, artificial powder diets that were partially defined were added to water to create a slurry before autoclaving (Pham, Kanther, Semova, & Rawls, 2008). Although these diets were consumed and evoked appropriate responses (Rawls et al., 2006), the impact of autoclaving and leaching on bioavailability and content, and potentially on water quality, was not studied in detail. To bypass these caveats, we next irradiated defined-composition, artificial powder diets (Semova et al., 2012). Despite presumed improvements in nutrient content and bioavailability, fish fed these diets display suboptimal, post-larval growth. While the zebrafish research community works to determine nutritional requirements, we focus our efforts on developing live diets for GF animals. Although it may prove to be more difficult to achieve reproducible nutritional content with live diets compared to artificial diets, preliminary studies indicate that live GF diets yield better growth. Several groups have succeeded in culturing GF zebrafish for up to a month, using diets based on *Tetrahymena* and *Artemia* (Milligan-Myhre et al., 2011; Rendueles et al., 2012). In the future, it will be important to refine protocols for reproducible culture of live GF diets and define their nutritional content, to more fully understand the impact of GF status on development and physiology. Broader efforts to define zebrafish nutritional requirements and development and implementation of a standardized artificial diet could be used as a platform for customized diets with targeted manipulations of specific nutrients. Only after these resources are established across zebrafish research will they be applicable to study zebrafish–microbiota interactions.

2.2 HOUSING

Use of tissue culture flasks or multiwell plates for rearing GF fish enables gnotobiotic zebrafish to be maintained outside of a gnotobiotic isolator, making GF husbandry accessible to laboratories that only have BSCs. However, scalability of growing gnotobiotic zebrafish in culture flasks and multiwell plates has limitations. The requirement for media changes increases daily husbandry labor and can become limiting as we strive to increase throughput and extend GF husbandry to adults.

We envision innovations that could help more easily and consistently rear gnotobiotic zebrafish through their life cycle. First, transitioning from flasks into bioreactor-like stand-alone units with various entry ports connected to reservoirs for dosing of fresh sterile media for water changes, feeding, and colonization. Units should also have outflow ports for used media, situated to prevent detritus accumulation. Similar vessels have already been developed for other fishes (Forberg et al., 2011; Lesel, 1979). Second, use of interconnected mini-bioreactors could permit large-scale screening of GF fish. This type of apparatus has already been developed for culturing microorganisms (Auchtung, Robinson, & Britton,

2015) and should be adaptable for zebrafish and other small aquatic animals. Together these proposed innovations could allow zebrafish to become the only gnotobiotic vertebrate model for which large-scale, long-term husbandry is possible.

2.3 POST-LARVAL GNOTOBIOTIC ZEBRAFISH

Our recent advances in gnotobiotic zebrafish husbandry allow us to begin studying later life cycle stages under GF conditions. This exciting frontier will greatly benefit from improved housing and diet. Although GF husbandry of post-embryonic stages remains challenging, we anticipate that advancing this part of the gnotobiotic zebra-fish field will empower analysis of these and other topics.

2.3.1 Metamorphosis and post-embryonic growth and development—Post-embryonic zebrafish development and growth are accompanied by metamorphosis, during which irreversible morphological and physiological changes occur as the animal transitions from larval to juvenile and adult forms (McMenamin & Parichy, 2013). The diversity of traits that undergo metamorphic changes is vast, yet, only a limited number of them have been studied (McMenamin & Parichy, 2013). Concomitant changes of the microbiota and animal form have been shown during metamorphosis in invertebrates, amphibians, and jawless fishes (Johnston & Rolff, 2015; Kohl, Cary, Karasov, & Dearing, 2013; Tetlock, Yost, Stavriniades, & Manzoni, 2012). Metamorphosis is tightly linked to the environment (Paris & Laudet, 2008). We previously reported that zebrafish intestinal microbiota changes during and after metamorphosis (Burns et al., 2016; Stephens et al., 2016; Wong et al., 2015), but it remains unclear whether metamorphic processes in zebrafish, or in other animals for that matter, are influenced by the microbiota.

2.3.2 Obesity and metabolic disease modeling—Zebrafish have been used to model genetic obesity (Song & Cone, 2007), body fat distribution (Minchin et al., 2015), and obesogenic molecules (Tingaud-Sequeira, Ouadah, & Babin, 2011). White adipose tissues develop in teleosts, but in zebrafish this tissue does not appear until around the onset of metamorphosis and then continues to increase in size and distribution as animals grow to adult stages (Flynn, Trent, & Rawls, 2009; Imrie & Sadler, 2010). Zebrafish white adipocytes have similar morphology and gene expression to mammalian adipocytes (Flynn et al., 2009; Imrie & Sadler, 2010), and gnotobiotic murine models have revealed microbial influences on adipose tissue accumulation and metabolic disease (Bäckhed et al., 2004; Cani et al., 2008; Ridaura et al., 2013). There is already evidence of microbiota contribution to intestinal fat absorption in zebrafish (Semova et al., 2012), and microbial modulation of host regulators of fat metabolism like *Angptl4* is conserved in mice and zebrafish (Bäckhed et al., 2004; Camp, Jazwa, Trent, & Rawls, 2012). In the future, use of post-larval GF zebrafish can complement and extend existing efforts to understand microbial contributions to fat storage and energy balance.

2.3.3 Immunity—During the first few weeks post fertilization, zebrafish lack an adaptive immune system and instead rely exclusively on the innate immune system to respond to immune challenges (Kanter & Rawls, 2010). Development of tissues and organs involved in adaptive immunity and the humoral response is observed about 4–6 weeks post

fertilization (Lam, Chua, Gong, Lam, & Sin, 2004; Langenau et al., 2004; Page et al., 2013). Zebrafish possess adaptive immune system cells, including B cells, T cells, and dendritic cells, however structures homologous to gut-associated lymphoid tissue and Peyer's patches have not yet been identified in teleosts (Renshaw & Trede, 2012). Both innate and adaptive arms of the immune system are required for normal host-microbe interactions, as demonstrated by the hypersusceptibility to infection of immunocompromised zebrafish lines lacking the innate immune signal transducer *myd88* or the adaptive immunity cell receptor recombinaise *rag1* (Swaim et al., 2006; van der Vaart, van Soest, Spaink, & Meijer, 2013). Extending the GF state to later life cycle stages should enable testing of microbial influences on development and function of zebrafish adaptive immunity, as has been shown in mammals (Honda & Littman, 2016). Thus, GF post-larval zebrafish could greatly contribute to comparative and evolutionary studies of vertebrate immune responses.

2.3.4 Xenobiotic metabolism—The gut microbiota are known to modulate interactions of the host with foreign substances or xenobiotics (Haiser, Seim, Balskus, & Turnbaugh, 2014; Saha, Butler, Neu, & Lindenbaum, 1983; Zheng et al., 2013) and to regulate differential expression of genes involved in xenobiotic metabolism in livers of GF and specific pathogen-free mice (Bjorkholm et al., 2009). However, the role of microbiota in xenobiotic metabolism remains under studied. Post-embryonic GF zebrafish could be a platform to investigate microbial-dependent changes of host-xenobiotic interactions, as well as host-microbiota interactions in the context of the response, adaption, and long-term consequences of exposure to xenobiotics, including therapeutic drugs and environmental chemicals. GF zebrafish as a toxicology model provide the ability to regulate the microbial environment and the nominal concentration of chemicals, along with regulation of the host genetic background, to understand how the microbiota and genetics intersect to influence host responses to xenobiotics.

2.3.5 Behavior—Zebrafish has become a premier model for exploring cellular and molecular mechanisms underlying a variety of behaviors, including swimming, as well as social, sexual, cognitive, sleep, reward, and anxiety (Fetcho, Higashijima, & McLean, 2008; Kalueff et al., 2013). All of these behaviors are generated by neural circuitry that can be readily studied through the course of development, making zebrafish an ideal platform to learn the role of the micro-biota in nervous system development and function. Coupling use of transgenic zebrafish in which morphology or activity of specific neuronal subpopulations can be visualized (Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014) with use of genetically modified bacteria (Stephens et al., 2015) will enable unprecedented resolution of molecular mechanisms by which resident microbes affect the nervous system and in turn, by which the nervous system affects resident microbial communities.

In summary, there are many exciting areas in which the well-established genetic and imaging zebrafish toolkit can be combined with gnotobiotic husbandry to investigate mechanisms by which resident microbiota influence host development, physiology, metabolism, and behavior.

3. SOLUTIONS

Here we list solutions required for each procedure.

1. Embryo medium (EM)

Stock solutions

- a. A: 250 mL RO H₂O, 20 g NaCl, 1 g KCl
- b. B: 100 mL RO H₂O, 0.358 g Na₂HPO₄ anhydrous, 0.6 g KH₂PO₄ monobasic
- c. C: 250 mL RO H₂O, 4.68 g CaCl₂ dihydrate
- d. D: 250 mL RO H₂O, 6.15 g MgSO₄·7H₂O
- e. E: 250 mL RO H₂O, 8.75 g NaHCO₃

Preparation (5205 mL)

- a. Add 5 L of RO water to a 5 L carboy
- b. Add 50 mL stock solution A
- c. Add 5 mL stock solution B
- d. Add 50 mL stock solution C
- e. Add 50 mL stock solution D
- f. Add 50 mL stock solution E
- g. Adjust pH to 7.2 with 1 M HCl

2. Antibiotic embryo medium (ABEM)

- a. 500 µL ampicillin (Fisher Scientific, BP1760-25) 100 mg/mL (100 µg/mL final)
- b. 50 µL kanamycin (Fisher Scientific, BP906-5) 50 mg/mL (5 µg/mL final)
- c. 15.6 µL amphotericin B (VWR, 97061-608) 8 mg/mL (250 ng/mL final)
- d. 500 mL EM
- e. Mix and filter sterilize with 0.2 µm filtration unit

3. 0.003% Bleach solution

- a. 125 µL 6.0% laboratory-grade bleach solution (Fisher Scientific, SS290-1)
- b. 250 mL EM
- c. Mix and filter sterilize with 0.2 µm filtration unit

4. 0.1% Polyvinylpyrrolidone-iodine (PVP-I) solution

- a. 2.5 mL 10% PVP-I stock (Ovadine, Western Chemical, Inc.) 5 g PVP-I in 50 mL nanopure water)
 - b. 247.5 mL EM
 - c. Mix and filter sterilize with 0.2 μ m filtration unit
5. Modified Neff's media (MNM)
 - a. Dissolve 3 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 500 mL RO water
Add
 - b. 10 g proteose peptone (VWR, CRITERION, Hardy Diagnostics, 89405-922)
 - c. 2.5 g yeast extract (Fisher BioReagents, BP9727-500)
 - d. 5 g glucose (Fisher Scientific, AC410950010)
 - e. Add RO water to 1 L
 - f. Autoclave
Caution: $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is corrosive and hygroscopic.
6. Proteose peptone yeast media (PPYE)
 - a. 0.75 g proteose peptone
 - b. 0.75 g yeast extract
 - c. Add RO water to 300 mL
 - d. Autoclave
 - e. Using sterile technique, dispense 15 mL media into 50 mL conical sterile tubes
7. Sterile 30% organic egg emulsion in embryo media
Materials
 - a. Organic chicken eggs
 - b. Micro-90 concentrated cleaning solution (VWR, 89210-138)
 - c. Double distilled water (dH_2O)
 - d. 1 L polypropylene beaker for egg soaking
 - e. Scotch-Brite abrasive (use once and dispose afterward)
 - f. 250 mL autoclaved beakers
 - g. 1 L autoclaved plastic waste beaker
 - h. 70% ethanol
 - i. Aluminum foil
 - j. $n \times 50$ mL conical tubes n = number of eggs

- k.** P-1000 sterile pipet tips
- l.** 250 mL sterile EM
- m.** 1 × 50 mL sterile wrapped serological pipet

Procedure

- 1.** Soak organic eggs in 1% Micro-90 concentrated cleaning solution in a 1 L plastic beaker for at least 3 h.
- 2.** Scrub shells of three eggs with an abrasive sponge.
- 3.** Rinse 3 times in dH₂O.
- 4.** Replace dH₂O with 70% EtOH, soak at least 1 h.
- 5.** Transfer each egg to a separate 250 mL beaker and cover with fresh 70% EtOH. Place foil over top of beaker.
- 6.** Clean the following with 70% EtOH and place in BSC:
 - a.** Large sheet of aluminum foil to protect surface of the BSC work surface. Do not block vents (*Note:* Egg is difficult to remove from stainless steel surfaces)
 - b.** Beakers containing eggs
 - c.** 1 × 1 L beaker (waste)
 - d.** n × 50 mL conical tubes n = number of eggs
 - e.** 250 mL sterile EM
- 7.** Label 50 mL conical tubes and loosen caps. Place upright in rack.
- 8.** Remove foil from one beaker. Carefully decant 70% EtOH into 1 L waste beaker.
- 9.** Place beaker on aluminum foil. Crack egg and separate egg white into beaker by passing the yolk from one half shell to the other. The goal is to have the intact yolk with very little white in one of the half shells. Discard shell without yolk.
- 10.** Holding the shell with intact yolk, use a sterile P-1000 pipet tip to pierce the yolk membrane. Gently tilt the shell to pour yolk out of membrane into a sterile 50 mL conical tube. Do not let membrane slide into conical tube, it does not emulsify. Put lid on conical tube.
- 11.** Discard shell and P-1000 tip into waste beaker.
- 12.** Spray hands and work with 70% EtOH. Change gloves as needed.
- 13.** Repeat yolk isolation process for remaining eggs.
- 14.** Using graduation on sides of 50 mL conical tubes, estimate volume of yolk collected. Calculate volume of sterile EM to add for a 30% solution.

15. Use pipet aid and 50 mL sterile wrapped serological pipet to dispense sterile EM 0o each conical tube containing egg yolk. Screw caps of tubes tightly and shake vigorously to emulsify.
16. Remove tubes of emulsion from BSC.
17. Incubate 30% egg yolk emulsion at 28°C for 24 h.
18. Plate 200 µL of emulsion on TSA plates. Spread evenly with sterile glass beads. Avoid bubble formation while spreading. Bubbles can look like bacterial colonies later.
19. Incubate plates at 30°C, check for bacterial growth at 24 and 48 h.

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FIGURE 1.

Overview of critical procedures for GF zebrafish derivation. Timeline depicts timing of procedures (see Tables 1–3 for specific details). Day refers to egg fertilization. Day –1: Adult fish are set up for breeding and tank water collected for conventionalization. Day 0: Adult zebrafish spawn and embryos are collected. If experiments demand conventional (CV) controls, embryos are segregated following collection and embryos destined for GF derivation are treated with antibiotics. Following GF derivation, embryos are placed in tissue culture flasks and conventionalized (CVZ) controls inoculated with a small amount of parental tank water collected on Day 0. Day 3: Flasks are inspected to ensure larvae have hatched; unhatched larvae are dechorionated by gently shaking the flask. Day 4: Flasks are visually inspected with phase optics to ensure sterility; Fig. 2 shows examples of contamination. Larval zebrafish can be kept in tissue culture flasks up to 8 dpf without feeding; for longer experiments axenic feeding and care should begin at 4–5 dpf.

FIGURE 2.

Phase microscopy visualization of potential flask contaminants and identification of sterile debris. Microorganisms present in tank water or CVZ flasks include bacteria (A, B *arrowheads*), filamentous microorganisms (A, *arrow*) and protozoans such as amoeba (B, *asterisk*). Reflective, irregularly shaped debris (*arrows*) observed in an otherwise sterile flask (C). Scale bar (A, C) 50 μ M,

FIGURE 3.

Microbial association of GF zebrafish procedure overview. Schematic diagram to be used with Tables 4 and 5 Number and letter sequences match procedures in Table 5. (A) Experiments with individual bacterial isolates to relate culture density to viable CFUs/mL to enable calculation of inoculation volume; see Table 5A for sample calculations. (B) For each experiment, grow and prepare bacterial inoculum according to specifications determined in (A). Pellet and wash bacteria with sterile EM, pellet again and suspend in sterile EM. Use washed bacteria for flask inoculation. (C) (1) Prior to inoculation, inspect flask for contamination under phase optics and remove media to plate any culturable bacterial contaminants. (2) Inoculate flask with washed bacteria and mix gently. Verify density by sampling flasks following inoculation (3) and at experimental end point (4).

FIGURE 4.

Long-term GF husbandry considerations. Caring for GF zebrafish on a continuum from embryonic stages to adulthood resembles the process for assembling a small fish facility. Developing housing, appropriate, stage-specific nutrition, and efficient media delivery and waste removal under sterile conditions are paramount.

FIGURE 5.

Tetrahymena culture maintenance overview. Schematic diagram to be used with Tables 8–10; letters in figure correspond specifically to steps in Table 8.

FIGURE 6.

Tetrahymena nutrient enhancement. (A) *Tetrahymena* cultured in proteose peptone medium are small and have few vacuoles. (B) *Tetrahymena* cultured in chicken egg yolk-enriched medium are larger and packed with large vacuoles (*arrow*). Scale bar, 20 μm .

Table 1

Example Zebrafish Embryo GF Derivation Time Table. Refer to Tables 2 and 3 for Materials and Specific Procedures. This Procedure Precedes Defined Microbial Associations and/or Long-term Husbandry Experiments

Day	Time	Procedure(s)
-1	4–5 p.m.	Adult zebrafish breeding preparation
0	8:30–9 a.m.	Adult zebrafish spawning
	9:30–10 a.m.	Embryo collection, rinse in ABEM
	11:00 a.m.	Sort embryos, rinse in ABEM
	1:00 p.m.	Sort embryos, rinse in ABEM
	2:30–3:30 p.m.	Disinfection solution, embryo, and biosafety cabinet preparation
	3:30–4:30 p.m.	Surface disinfection procedure in biosafety cabinet
	4:30 p.m.	Conventionalize control embryos
1	Any time	Health check
2	Any time	Health check
3	Any time	Dechorionate unhatched larvae, health check
4	Morning	Health check, visual inspection with phase optics, culture flask media

Table 2

GF Derivation Materials and Solutions. Refer to Table 1 for Example Time Schedule and Table 3 for Specific Procedures. Solution Recipes Follow Chapter. Prepare Shaded Solutions Immediately Prior to Use

Item(s)/Solutions	Special Considerations
Sterile screw top tissue culture flasks with filter caps	Short-term experiments: 25 cm ² (15 mL with 10–15 embryos) or 75 cm ² (50 mL with 25–50 embryos)
	Long-term experiments: 150 cm ² (100 mL with 20–25 embryos)
50 mL glass beakers	Bleach, rinse, autoclave with foil covers
	2 beakers for every 200 eggs disinfected
Individually wrapped sterile transfer pipets	
25 or 50 mL wrapped, sterile serological pipets	
1–2 L beaker	
Pipet aid	
70% ethanol (EtOH) in spray bottle	
6% bleach, laboratory grade	Store at 4°C, replace every 3 months
10% polyvinylpyrrolidone (PVP-I)	Store in dark, replace every 3 months
100 × 20 mm polystyrene petri dishes	
Sterile gowns or sleeves	Wear when working in BSC
0.2 µM filtration units, 1 L, 250 mL	Clean aspiration tubing regularly to prevent backflow contamination
Sterile Embryo Medium (EM)	Make in advance
Antibiotic embryo medium (ABEM)	Make just prior to egg collection, filter sterilize
0.003% bleach solution	Make both just prior to surface sterilization, filter sterilize
0.1% PVP-I solution	
Digital timer	

Table 3

GF Derivation Procedure. Example Schedule and Materials Found in Tables 1 and 2, Respectively. Solution Recipes Follow Chapter. Perform Steps 11–24 Using Sterile Technique With Attention to BSC Function. Shading Differentiates Days in the Procedure Sequence

Day	Procedure
–1	<ol style="list-style-type: none"> 1 Set up adult zebrafish overnight in breeding cages with dividers to prevent natural spawning. 2 Collect parental tank water for conventionalizing embryos into a sterile bottle. Incubate at 28°C overnight.
0	<ol style="list-style-type: none"> 3 Transfer adult fish into clean breeding cages with fresh, autoclaved, or sterile filtered system water. Remove dividers to allow for breeding. Allow less than 1 h to ensure that embryos are of similar ages. 4 Collect embryos, thoroughly rinse to remove visible debris. 5 Transfer embryos to petri dishes with fresh, sterile ABEM, ~1000 embryos per dish. 6 Divide embryos further into 3–4 petri dishes with fresh ABEM, remove any debris. 7 Incubate embryos at 28–30°C between 4 and 6 h depending on temperature. 8 At ~2 h intervals, remove infertile embryos, transfer fertile embryos to clean dishes with fresh ABEM, ending with ~200 healthy embryos per dish. 9 At 50% epiboly up to shield stage (see Kimmel et al., 1995), transfer up to 200 viable embryos into foil-covered, autoclaved 50 mL beakers with ~40 mL of ABEM. Replace foil cover. 10 Prepare biosafety cabinet (BSC) for use. Put on gloves and gown or sterile sleeves, clean hands and arms thoroughly with 70% ethanol (EtOH). 11 Clean the following with 70% EtOH. Do not block BSC vents. <ol style="list-style-type: none"> a. 50 mL sterile beakers with foil tops b. Individually wrapped sterile transfer pipets c. 1 L filter-sterilized EM d. 0.003% bleach solution e. 0.1% PVP-I solution f. Sterile flasks with filter screw caps of desired size g. 25 or 50 mL wrapped, sterile serological pipets h. Large beaker (1–2 L) for waste collection i. Pipet aid dedicated to BSC use j. Foil-covered beakers holding embryos. Do not allow ethanol seepage into beakers. 12 Remove foil top from embryo beaker, slowly decant liquid into waste beaker. 13 Rinse 3 times with sterile EM (<i>Note:</i> Do not use ABEM). <ol style="list-style-type: none"> a. Add ~40 mL sterile EM to beakers of embryos. Allow embryos to settle. b. Decant liquid into waste beaker, reserve embryos. c. Add ~40 mL sterile EM. d. After final rinse, decant all liquid into waste beaker. 14 Immerse embryos in ~50 mL sterile 0.1% PVP-I for <i>exactly</i> 2 min. 15 Decant 0.1% PVP-I solution into waste beaker. 16 Rinse embryos 3 times with sterile EM as described in steps 13a–d. 17 Transfer embryos into new sterile 50 mL beaker. <ol style="list-style-type: none"> a. Add ~20 mL sterile EM to beaker of embryos. b. Gently swirl embryos to resuspend, pour embryos into new sterile 50 mL beaker. c. Repeat a and b to collect any remaining embryos. Do not overfill beakers.

Day	Procedure
	<p>18 Allow embryos to settle, decant liquid into waste beaker, reserve embryos.</p> <p>19 Immerse embryos in ~50 mL 0.003% sterile bleach for 18–20 min.</p> <p>20 During bleach immersion, fill flasks with recommended volumes of sterile EM.</p> <ul style="list-style-type: none"> a. Using pipet aid and sterile serological pipets, dispense sterile EM into flasks. b. For short-term experiments, dispense 13 mL sterile EM into 25 cm² flasks or 48 mL sterile EM into 75 cm² flasks. c. For long-term husbandry, dispense 98 mL sterile EM into 150 cm² flasks. <p>21 Following bleach immersion, decant bleach into waste beaker.</p> <p>22 Rinse embryos 3 times with sterile EM as in steps 13a–c.</p> <p>23 Transfer embryos into flasks of sterile EM using sterile transfer pipets.</p> <ul style="list-style-type: none"> a. 10–15 embryos per 25 cm² flasks. b. 25–50 embryos per 50 cm² flasks. c. 20–25 embryos per 150 cm² flasks. <p>24 Tighten lids on all flasks and remove from BSC.</p> <p>25 Inoculate control flasks with reserved parental tank water.</p> <p>26 Incubate embryo flasks at 28°C with a 14 h light/10 h dark cycle.</p>
1–2	<p>27 Perform health check, noting sick or dead embryos.</p>
3	<p>28 Mechanically dechorionate unhatched larvae by flicking flask to knock embryos against the side of the container.</p> <p>29 Perform health check, noting sick or dead embryos.</p>
4	<p>30 Perform health check, noting sick or dead embryos.</p> <p>31 Begin feeding and/or microbial associations as experimental paradigm requires.</p>

Table 4**Bacterial Association of GF Zebrafish Materials**

Material	Special Considerations
Bacterial strain of choice	Consider biosafety level, determine growth characteristics and optimal inoculum in advance
Liquid growth media	Appropriate for strain used
Sterile EM	Bacteria must be washed and suspended in EM prior to zebrafish association
Tryptic soy, LB, or BHI agar plates	Make in advance
Sterile test tubes with lids	Autoclave prior to use
Sterile 1.6 mL tubes	
Sterile plating beads	

Table 5

Microbial Association of GF Zebrafish Procedure. *Do Not* Perform Bacterial Work in BSC Dedicated to GF Derivation. Part A is Repeated Several Times for Each Bacterial Strain to Ensure Reproducibility Prior to an Experiment. Shading Indicates Separate Procedures. Numbered Sequences for Each Procedure Also Appear in Fig. 3

Part	Procedure	Instructions
A	Initial assessment of bacterial isolate growth characteristics	<p>Determine the concentration of <i>viable</i> cells in overnight cultures for each new strain of bacteria used to calculate the experimental dilution volume for inoculation reproducibly.</p> <ol style="list-style-type: none"> 1 Measure the OD₆₀₀ of an overnight culture. 2 Perform serial dilutions, plating and enumerate colony growth. 3 Calculate the culture density. <p>Example: For <i>Escherichia coli</i>, an OD₆₀₀ of 1 typically indicates that the concentration of viable cells is 10⁹ colony-forming units (CFU/mL). <i>Note</i>:</p> <p>Sample calculation for <i>Aeromonas</i> overnight culture:</p> $OD \times \text{dilution factor} \times \text{conversion factor} = \text{CFU/mL}$ $0.765 \times 10 \times (1 \times 10^9) = 7.65 \times 10^9 \text{ CFU/mL}$ <ol style="list-style-type: none"> 4 Calculate inoculation volume. <p>Example: For <i>Aeromonas</i> inoculation in flask with 15 mL sterile EM at 1 × 10⁶/mL:</p> $(C_1)(V_1) = (C_2)(V_2)$ $(7.65 \times 10^9 \text{ CFU/mL})(X \text{ mL}) = (1 \times 10^6 \text{ CFU/mL})(15 \text{ mL})$ $x = 0.00196 \text{ mL} = 1.96 \mu\text{L}$
B	Growth and preparation of bacterial inoculum	<ol style="list-style-type: none"> 1 Incubate bacteria overnight (~16 h) in 5 mL of growth medium in a 20 mL test tube with shaking at 28–30°C. 2 Wash bacteria prior to association with zebrafish larvae. <ol style="list-style-type: none"> a. Pellet 1 mL of an overnight culture for 2 min at 7000xG. b. Discard supernatant by aspiration. c. Gently suspend pellet in 1 mL sterile EM by pipetting. d. Pellet for 2 min at 7000xG. e. Discard supernatant by aspiration. f. Gently suspend pellet in 1 mL sterile EM by pipetting.
C	Sterility verification, flask inoculation, bacterial density verification	<p>In the following steps, minimize opening/closing of flask to reduce contamination risk. For consistency, we recommend dispensing small inoculation volumes by suspending them in 1 mL sterile EM and transferring this suspension to flasks using a 1 mL pipet. It is important to also maintain flask volume by first removing an equivalent volume of water from flasks (i.e., 1 mL).</p> <ol style="list-style-type: none"> 1 Sterility verification <ol style="list-style-type: none"> a. Inspect flasks using phase optics for bacterial contamination. b. Using sterile technique, remove 500 µL of media from each flask. c. Spread 100 µL of each tube in step b on TSA or LB plates with sterile beads. d. Incubate for 48 h. e. Inspect plates for growth of bacterial contaminants. 2 Inoculate flask with calculated dilution of washed bacteria determined in step A4 to flask aseptically (e.g., 1.96 µL washed <i>Aeromonas</i> cells suspended in 1 mL sterile EM). 3 Sample initial inoculation level by removing 500 µL from flask and adding to sterile EM in 1.6 mL tube:

Part	Procedure	Instructions
		<div><div>a. Perform serial dilutions of sample in sterile EM.</div><div>b. Plate 100 µL from each dilution on TSA or LB plates.</div><div>c. Incubate 24 h at 30°C.</div><div>d. Count CFUs and calculate inoculation level.</div></div> <div>4 Sample experimental end point bacterial density by repeating step 3.</div>

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Table 6

Additional Materials for Long-Term GF Husbandry. These Materials Supplement Those for GF Derivation. Refer to Tables 1–3 for GF Derivation Timeline, Materials and Procedures. Refer to Long-term Husbandry Procedures in Table 7. Solution Recipes Follow Chapter

Material	Special Considerations
Controlled temperature room or incubator (28°C) with 14 h light/10 h dark cycle	Control clutter, clean work spaces with 70% ethanol following use, empty trash daily
Filter-sterilized EM	Enough for 50–70% daily water exchange, verify sterility by plating on TSA prior to use
Sterile food cultures	Verify sterility by culturing on TSA prior to use
2 L autoclaved waste beakers	Separate beakers for GF and CVZ, cover with foil and autoclave prior to use
15 and 50 mL sterile conical tubes and rack	Autoclave racks regularly
Ethanol-resistant pens	Labeling tubes and plates
GF dedicated pipet aid, P-1000 and P-200	Keep in biosafety cabinet, clean with 70% ethanol following use
CVZ dedicated pipet aid, P-1000, P-200	Store away from GF supplies, clean with 70% ethanol following use
Sterile filter tips for P-1000 and P-200	Dedicated sets for GF and CVZ
Sterile 50 mL and 10 mL wrapped serological pipets	Used for media exchange, water sample collection, and removing dead larvae
Ammonia test kit (API, item# LR8600)	Follow manufacturer's instructions, 5 min incubation is critical
Trays	Use separate trays for storing GF and CVZ flasks
Daily data sheets	Record feeding volume, plating results, dead fish numbers, summary sheet
Dissecting scope	Clean stage and focus knobs with 70% ethanol following use
Phase contrast inverted microscope	
TSA plates	Have at least 2 days' supply ready in advance
Sterile plating beads	Autoclave before use

Table 7

Current Long-Term GF Husbandry Procedures. Follow Procedure for GF Derivation of Embryos (Table 3). Live Food Culture Materials and Procedures Follow in Tables 8–10 Solution Recipes Follow Chapter. Beginning on Day 4, Continue With Long-term Husbandry Morning and Afternoon Procedures Outlined in the Following. This Procedure Includes Both GF and CVZ Conditions. All GF Procedures Should be Performed Using Sterile Technique in BSC. Record Data Recommended in Text and Table 6 Daily and Prepare Sterile Media, Food Cultures and TSA Plates in Advance. Table Shading Indicates Different Procedures.

Important: Verify Sterility of all Solutions Prior to Use

Procedure	a.m.	p.m.	Instructions
Check cultures	✓;		<ol style="list-style-type: none"> 1 Check bacterial plates to verify sterility of reagents and GF flask media. 2 Get fresh TSA plates out of refrigerator and warm to 28°C.
Health check	✓;	✓;	<ol style="list-style-type: none"> 3 Health check <ol style="list-style-type: none"> a. Inspect each flask, record fatalities. b. Remove inviable larvae from CVZ flasks only. Do not compromise sterility of GF flasks.
Visual inspection for contamination	✓;		<ol style="list-style-type: none"> 4 Visual inspection <ol style="list-style-type: none"> a. Inspect a CVZ flask with phase optics focusing on bacteria adhering to the bottom (see Fig. 2). Motile bacteria should be visible on the bottom and in the water column. b. Inspect GF flasks at the same focal plane as CVZ flask. Record results for each flask in data table and in notes. c. If bacteria are visible in GF flasks, remove flask from experiment. In ambiguous cases, segregate questionable flasks until sterility is determined.
Media exchange/water sampling	✓;	✓; (CV/CVZ only)	<ol style="list-style-type: none"> 5 General guidelines for media exchange and water sampling. <i>Note:</i> Larvae are sensitive to turbulence and handling. <ol style="list-style-type: none"> a. Handle GF samples in BSC. Clean with 70% EtOH prior to entry, do not overcrowd workspace. Divide flasks into manageable groups for media exchange and feeding. b. Handle CV/CVZ samples in dedicated space. c. On Days 4–6 only remove small amounts of media for sampling. add ~50 mL sterile EM daily to dilute ammonia waste build up. 6 Waste media removal beginning 7 dpf. <ol style="list-style-type: none"> a. With a sterile pipet, remove 50–100 mL media from flasks. Replace lid, move flask out of the way. b. Pipet 5 mL of the removed volume into 50 mL conical tube for cumulative ammonia testing. c. Pipet 5 mL into a 15 mL conical tube for visual inspection or plating, replace lid. d. Pipet remaining liquid from media removal into large waste beaker. e. Repeat a–d for each flask.

Procedure	a.m.	p.m.	Instructions
Media addition	✓;	✓; (CV/CVZ only)	<p>7 Fresh media addition (begin day 4)</p> <p>a. Using a sterile 50 mL pipet, add sterile EM to match volume removed to each flask.</p> <p>b. Add fresh media to all flasks in BSC before feeding.</p>
Feeding	✓;	✓; (CV/CVZ only)	<p>The volume of live food added depends on <i>Tetrahymena</i> culture density, age of fish, volume of media exchange, and overall volume of media in flask. Check <i>Tetrahymena</i> density by viewing flask on dissecting scope following feeding.</p> <p>8 Feeding</p> <p>a. Using a P-1000 with sterile filter tip, add 48 h, sterile 1% egg yolk + <i>Tetrahymena</i> culture to each flask. Replace lid and tighten.</p> <p>b. From Days 1–7, place flasks upright on trays for storage. Once volume reaches 250 mL, incubate flasks on side for better air exchange.</p>
Ammonia testing	✓;		<p>9 Ammonia testing</p> <p>a. Perform ammonia testing on both cumulative GF and CVZ waste media according to manufacturer's instructions. Incubate colorimetric test for full 5 min.</p> <p>b. Ammonia levels must remain near 0 for optimal health.</p>
Flask media plating	✓;		<p>10 Be aware of air flow in room that might blow contaminants onto plates. Use sterile benchtop technique.</p> <p>11 Pipette 200 µL of each sample below onto TSA plates and spread evenly with glass beads.</p> <p>12 Negative control sterile glass beads</p> <p>b. Positive control 1–3 CV/CVZ flasks</p> <p>c. Individual GF flasks</p> <p>d. Food cultures</p> <p>e. Sterile EM (test sterility prior to use)</p>
Clean up	✓;	✓;	<p>12 Maintain clean work environment to reduce widespread contamination.</p> <p>a. Empty trash. Remove dirty glassware for cleaning.</p> <p>b. Sweep or mop floor, if needed.</p> <p>c. Clean all work surfaces and microscope stages with 70% ethanol.</p> <p>d. Spray door and cabinet handles.</p> <p>e. Clean sink area by routinely treating with 10% bleach, rinsing and then spraying with 70% ethanol.</p> <p>f. Refill 70% ethanol spray bottles.</p> <p>g. Discard bacterial plates in biohazard waste.</p>
Biosafety cabinet preparation	✓;	✓;	<p>13 Morning set up for afternoon session (food culture preparation). Clean with 70% ethanol and place in BSC.</p>

Procedure	a.m.	p.m.	Instructions	
			<div><div>a. 2 × 50 mL sterile conical tubes</div><div>b. 2 × 50 mL wrapped, sterile serological pipets</div><div>c. 250 mL bottle of validated sterile EM</div><div>d. PPYE + <i>Tetrahymena</i> (4–7 days old)</div><div>e. Aluminum foil to cover <i>Tetrahymena</i> cultures</div></div> <div>14Afternoon set up for morning session (media exchange and feeding). Clean with 70% ethanol and place in BSC.</div> <div><div>a. Sterile EM for media exchange.</div><div>b. 50 mL conical tube for ammonia testing of media removed.</div><div>c. 15 mL conical tubes for bacterial surveillance of media removed.</div><div>d. Wrapped sterile, serological pipets for media exchange.</div></div>	

Table 8

Quick Guide for *Tetrahymena thermophila* Live Food Culture. Refer to Fig. 5 for Culture Maintenance Schematic. Refer to Tables 9 and 10 for Materials and Specific Procedures. Solution Recipes can be Found at the end of the Chapter. Shading for Visual Separation of Reagents and Incubation Details

Corresponding Element in Fig. 5	B	C	D	E	F	G
Media	Original culture 5 mL MNM	Maintenance culture 5 mL MNM	Expansion culture 15 mL PPYE	Nutrient-loaded culture 37 mL Sterile EM	Sterility verification TSA	Zebrafish flask 100–250 mL Sterile EM
Penicillin- streptomycin cocktail (Sigma P4458)	5 µL	5 µL	10 µL	–	–	–
Amphotericin B (8 mg/mL)	1 µL	1 µL	–	–	–	–
30% sterile egg yolk emulsion	–	–	–	1.4 mL	–	–
<i>Tetrahymena</i> Inoculation (see Fig. 5A, Table 9)	100 µL Stock center aliquot	100 µL Original culture	200 µL Maintenance Culture	2 mL Expansion culture	200 µL Nutrient-loaded culture	Nutrient-loaded culture volume added varies with zebrafish stage, media exchange and media volume
Incubation temperature	20°C	20°C	28°C	28°C	28–30°C	28°C
Incubation duration	1 week	2–4 weeks between new cultures	4–7 days	1 day—plate to verify sterility 2 days—feed if sterile	2 days	Add sterile nutrient-loaded culture daily

Table 9

Tetrahymena thermophila Culture Materials. Refer to Fig. 5 and Table 8 for Quick Guide and Table 10 for Specific Procedure. Solution Recipes Follow Chapter

Materials	Special Considerations
<i>Tetrahymena thermophila</i> stock	<i>Tetrahymena</i> Stock Center, Cornell University (stock ID SD00178, strain CU428), verify sterility
Penicillin–streptomycin cocktail (pen/strep)	Sigma–Aldrich, P4458
Amphotericin B	VWR, 97061-608 make 8 mg/mL stock solution in DMSO
Sterile glass culture test tubes with lids	Autoclave
Sterile 50 mL conical tubes	
Pipet aid	Dedicated to GF procedures
50 mL sterile wrapped serological pipets	
P-20, P-100, P-1000 pipettors and sterile filter tips	Dedicated to GF procedures
Sterile Modified Neff's Media (MNM)	
Sterile proteose peptone yeast extract (PPYE) media	
Sterile 30% organic egg emulsion in EM	Verify sterility of both solutions prior to use
Sterile EM	

Table 10

Tetrahymena thermophila Culture Procedures. Refer to Fig. 5 and Table 8 for Quick Guide and Table 9 for Materials. Solution Recipes Follow Chapter.

Use Sterile Technique When Working With Tetrahymena Stocks and Verify Sterility Regularly. Pipette Tetrahymena inoculum From Surface of Culture. Constant Shaking of Cultures is Unnecessary. Table Shading Indicates Different Procedures

<i>Tetrahymena</i> culture type	Procedure
Initiation	<p>Inoculate four maintenance stocks upon receipt of the stock culture and four more 4 days later for a total of eight maintenance stocks using the procedure given as follows:</p> <ol style="list-style-type: none"> 1 To each glass test tube with lid, add 5 mL of sterile MNM, 5 μL of pen/strep cocktail and 1 μL of amphotericin B stock solutions using sterile technique at a laboratory bench. Shake tube gently from side to side to mix well. 2 Inoculate media with 100 μL of the purchased <i>Tetrahymena</i> stock. 3 Incubate stock culture tubes on benchtop at room temperature (~20°C) out of direct light. 4 Incubate maintenance stock at room temperature on benchtop for 1 week. Inspect tube for <i>Tetrahymena</i> growth. Plate 200 μL of each stock tube on TSA plates for 48 h to test for bacterial growth and visually inspect tubes of <i>Tetrahymena</i> for cloudy appearance indicating bacterial growth.
Maintenance	<p>This stock is kept in the laboratory at room temperature away from the fish facility and serially propagated for continued use to inoculate expansion cultures.</p> <ol style="list-style-type: none"> 1 Propagate maintenance stocks by serially transferring 100 μL of MNM culture to 5 mL of new, sterile media plus 5 μL pen/strep and 1 μL amphotericin B every 2–4 weeks. 2 Incubate maintenance stocks at room temperature for at least 1 week prior to use in expansion stocks.
Expansion	<p>Inoculate expansion culture on the benchtop and incubate for at least 4 days at 28°C prior to use for nutrient-enriched cultures.</p> <ol style="list-style-type: none"> 1 Aliquot 15 mL sterile PPYE into 50 mL conical tube. 2 Add 10 μL pen/strep to 15 mL of sterile PPYE using benchtop sterile technique. 3 Inoculate PPYE + pen/strep with 200 μL 1–2-week-old MNM culture. Leave lid slightly loose for air exchange. 4 Sample for bacterial growth before and after inoculation of the nutrient-loaded culture.
Nutrient loaded	<p>During an experiment, inoculate two cultures each day.</p> <ol style="list-style-type: none"> 1 Prepare nutrient-loaded culture in BSC. Wear gown or sterile sleeves, clean gloved hands and arms with 70% EtOH. 2 Clean the following with 70% EtOH thoroughly, and place in BSC. <ol style="list-style-type: none"> a. 50 mL conical tube of 30% egg yolk emulsion b. 250 mL filter-sterilized EM c. 4–7-day-old PPYE <i>Tetrahymena</i> culture d. 25 mL wrapped sterile serological pipet e. 2 $\times$ 50 mL sterile conical tubes (one for GF, one for CVZ culture) f. 400 mL beaker (waste) g. Foil large enough to wrap around 50 mL conical tube (if using UV in BSC) 3 Using serological pipet, fill each 50 mL sterile conical tube with 37 mL sterile EM. 4 Add 1.4 mL 30% sterile egg yolk emulsion to each tube. 5 Add 2.0 mL 4–7-day-old PPYE <i>Tetrahymena</i> culture. 6 Swirl tubes gently to mix.

<i>Tetrahymena</i> culture type	Procedure
	7 Wrap the GF-dedicated <i>Tetrahymena</i> culture in aluminum foil to protect from UV irradiation.
	8 Incubate cultures at 28°C. Sample for sterility after 24 h incubation. Use sterile 48 h cultures for feeding.

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