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Individual identification and marking techniques for zebrafish

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Abstract In laboratory fish research, the zebrafish *Danio rerio* (Cyprinidae) represents the equivalent of the mouse in mammalian research. This species has become a major model for studies in developmental and behavioural genetics, neurophysiology, biomedicine, ecotoxicology, and behavioural and evolutionary ecology. To meet the need for accurate and reproducible data in both fundamental and applied sciences, it is of primary importance to be able to tag and/or recognize individual zebrafish. However, classic methods used in fish ecology and aquaculture are generally difficult to apply to such small fish. Recently, various new tagging methods have been developed. This paper presents a first review of current identification and marking methods applied to zebrafish, from external observation methods (such as skin pattern recognition, fin clipping, scale regeneration, colour and transgenic methods) to the most advanced technological developments in electronic (low- and high- radio-frequencies PIT tags, microchip) and image analysis methods (video tracking). This review aims to help researchers and zebrafish facility managers select the identification method (ID) best adapted to their needs. The main characteristics of each ID method are examined (including detection range, durability, speed and repetitiveness, ID code combination, size dependence and ethical considerations), and their pros and cons are summarized in a decision table to help select the most appropriate option for a research or management program. Finally, contextual applications of these ID methods and future developments are discussed.

Keywords animal ID · *Danio rerio* · Passive Integrated Transponder · tagging · video tracking · VIE tag

Introduction

While numerous fish species have been used as biological models (Schartl 2014), the zebrafish (*Danio rerio*, Cyprinidae) has become the most commonly used model in laboratory research, covering disciplines as varied as genetics, developmental biology, oncology, toxicology, reproduction biology, neurobiology, ethology, environmental sciences, sociology and evolutionary biology (Lawrence 2007; Parichy 2006). Zebrafish present several major advantages compared to rodents. In particular, they require less space per subject, and are significantly cheaper to maintain and breed in a laboratory. After reaching maturity at the age of 3-4 months, a female can produce 200-300 embryos on a weekly basis. Furthermore, these embryos are translucent and rapidly develop outside of the mother's body, while requiring no parental care. This allows direct observation of the internal development of living embryos and larvae under a stereoscope, making them easily accessible for genetic and embryological manipulation (Lawrence 2007; Harper & Lawrence 2011).

The possibility of using large numbers of small-sized (even as adults) individuals makes this species very useful for screening purposes in genetic (Gerlai 2015), developmental (Tucci & Gerlai 2017), behavioural (Gerlai 2015; Kalueff 2017), neurobiological (Stewart et al. 2014a), toxicological (Hill et al. 2005; Raldúa & Piña 2014) and pharmaceutical research (Gibert et al. 2013, Mcgrath & Seng 2013; Veinotte et al. 2014). Their swimming behaviours are analysed and used in toxicological studies to diagnose potential aquatic toxicity of natural or drinking water, as they are sensitive enough to detect pollutants at concentrations that are very low but nevertheless problematic to human and environmental health (Magalhães et al. 2007; Huang et al. 2014; Oliva Teles et al. 2015). Their shoaling behaviours help psychologists and ethologists to better understand social interactions (Miller & Gerlai 2007, 2011, 2012) and cognition (i.e. social preferences, social learning, social recognition and social decision-making [Oliveira 2013]).

To meet the high research demands for this fish, numerous zebrafish facilities have been created around the world. Currently, the majority of animals used in laboratories come from official breeding structures. The fish are characterized by their strain as well as by any present mutation or transgene. However, nowadays, it has become increasingly necessary to identify individuals in order to track them throughout their lifetime, making it possible to follow their history, health and performance. This ability would refine the use of zebrafish in various scientific disciplines. First, it would allow breeding programs to easily pinpoint individuals with better reproductive performance, as well as track the degree of relatedness between individuals, thus increasing the quality of partner choices. Second, the screening of potentially interesting individual behaviours would become more efficient. For instance, the detection of less social individuals could serve as the basis to develop a new behavioural strain for the study of the causes and treatment of autism (Kalueff et al. 2014; Stewart et al. 2014b). Third, individual tracking allows the monitoring of each animal's health; for instance, by regularly evaluating weight or body condition, as well as the recovery of an individual that experienced a health problem or treatment. Fourth, in behavioural research, the ability to identify individuals is of primary importance in order to ascertain specializations or tactics, but also to evaluate the progress of each individual's performances (for example, to evaluate how learning improves with experience) (Martin & Bateson 2007; Brown et al. 2011). Finally, in research relying on collective behaviour, shoals are a model often used to understand processes of decision-making, transmission and sharing of information, as well as social learning (Brown et al. 2011; Sumpter 2010; Miller et al. 2013; Delcourt et al. 2016, 2018).

However, it is difficult to individually identify zebrafish due to their phenotypic similarity and because many tagging methods traditionally used in aquaculture and fisheries

cannot be applied to zebrafish due to their small size (Skalski et al. 2009; McKenzie et al. 2012). It is therefore essential to explore the most efficient ways to allow their individual identification.

The aim of this paper is to review the most practical individual identification methods used or potentially available for research on zebrafish, ranging from visual methods, such as the examination of natural skin patterns, to the most recent technological methods, such as Radio Frequency Identification (RFID) and video tracking. This review illustrates the diversity of methods developed in recent years to achieve individual identification for such a small fish species in the context of laboratory management (e.g. maintenance and stock breeding) and experimental research. More broadly, the transferability of these methodologies to other similar-sized fish species is also presented. Finally, guidelines are provided for the adequate choice of identification methods depending on the research question. Our review is focused mainly on the juvenile and adult zebrafish, as methods to identify individual larvae have been little explored.

Skin pigmentation and pattern

Zebrafish are easily recognized by their colour patterns: typically, a succession of 4 or 5 blue horizontal stripes alternating with 4 golden-silvered ones, giving the striking "zebra" pattern from which the species derives its vernacular name. Males and females have a similar colouration and lack of any obvious secondary sexual traits, often making it difficult for human observers to discriminate sexes. Hutter et al. (2012) have demonstrated slight sex differences in zebrafish, based on hue, saturation and brightness of stripes. The light stripes appear more silvery in females, whereas they seem more yellowish in males. Males' ventral fins also appear more yellowish; and the dark stripes on their flanks are somewhat darker than females' (Laale 1977; Schilling 2002; Hutter et al. 2011). These differences are more noticeable during courtship and reproduction, with males being more colourful and conspicuous (Hutter et al. 2010, 2012).

Three types of pigment cells (chromatophores) form the basis of the zebrafish's body patterns: melanophores (dark), xanthophores (gold) and iridophores (silvery/blue) (Kelsh et al. 1996; Singh et al. 2014; Mahalwar et al. 2014, 2016). The light stripes are composed of dense xanthophores over dense silvery iridophores, whereas the dark stripes are composed of a dense layer of melanophores loosely covered by blue iridophores (Singh et al. 2014; Mahalwar et al. 2014, 2016); Fig.1). The embryo and early larval colour pattern (stripes of melanophores at the edges of the myotomes and at the horizontal myoseptum, with a few iridophores within these stripes, and xanthophores scattered widely over the body) develops directly from neural crest cells (Patterson & Parichy 2013). The juvenile (and adult) pattern is acquired during the period of metamorphosis (20 to 45 days post-fertilization) from post-embryonic latent cells (Mahalwar et al. 2014; Parichy 2006). At the end of this period, two dark lateral stripes border a light interstripe. During growth, stripes and interstripes appear dorsally and ventrally (Patterson & Parichy 2013). In some mutants, the initial striped pattern observed in early juveniles can change into more complex designs during the juvenile-to-adult transition; in this case, the initial juvenile pattern disappears (Watanabe & Kondo 2015).

Based on mutants, chimeric individuals and laser ablation experiments, recent studies have shown that the three chromatophore types are required to form the striped pattern of the trunk, whereas only melanophores and xanthophores are required in the fins (Patterson & Parichy 2013; Frohnhöfer et al. 2013; Singh & Nüsslein-Volhard 2015). This skin pattern is a self-organising system based on short and long-range interactions between cells, known as a Turing-type system (Turing, 1952). During juvenile development, migration and differentiation of chromatophore cells are influenced by the previous presence or absence of other types of chromatophores. Interactions have positive or negative effects depending on the distance

between cell types (Frohnhöfer et al. 2013; Singh & Nüsslein-Volhard 2015). Absence or variations in magnitude of these interactions can generate a diversity of colour patterns in zebrafish (Fig. 2).

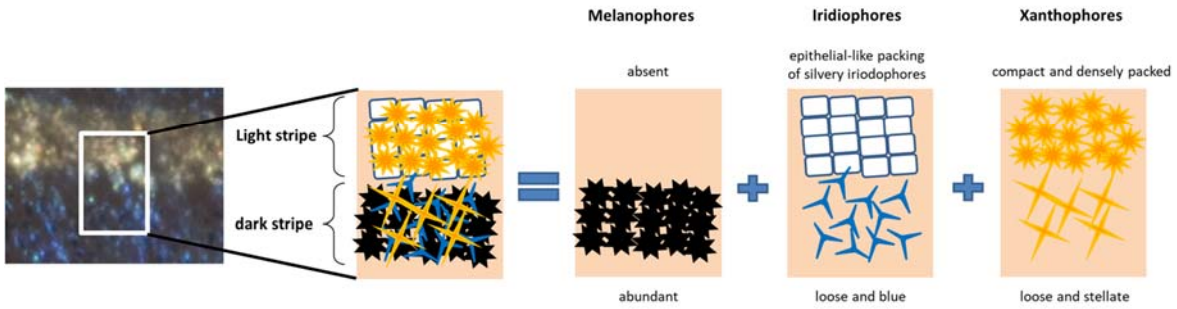


Fig. 1 Chromatophore composition of zebrafish skin in light and dark stripes (redrawn largely from Malahwar et al. 2016). Light stripes are composed of a dense, silvery iridophore layer covered by dense xanthophores, whereas dark stripes are composed of a dense melanophore layer loosely covered by blue iridophores and stellated xanthophores.

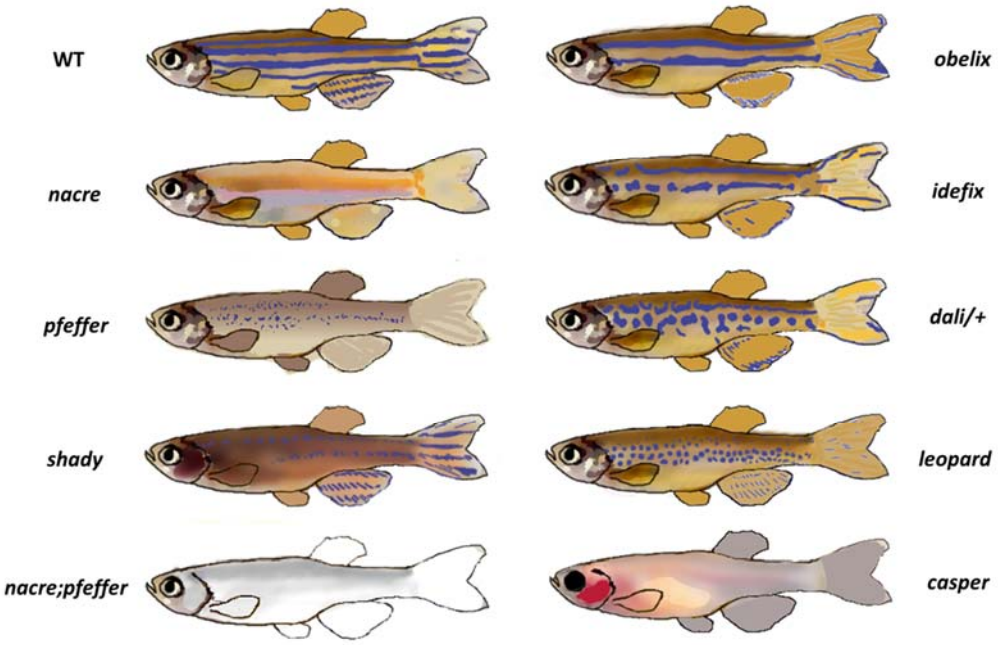


Fig. 2 Some schematic examples of the diversity of colour and pattern mutants in zebrafish. *nacre, pfeffer* and *shady* are mutants lacking respectively, melanophores, xanthophores, and iridophores with few melanophores. Among the double mutants, *nacre;pfeffer* are completely covered by dense iridophores; whereas *casper* (*roy;nacre*) are completely depigmented and translucent. Others are mutants of the stripe pattern: *obelix* has fewer and larger stripes than the wild type, *idefix* has fewer, narrower stripes which are frequently interrupted; *dali/+* has regularly-interrupted stripes with irregular orientations; *leopard* has a spotted pigmentation. Drawings are inspired from pictures published in Rawls et al. 2001, Irion et al. 2014, Engeszer et al. 2008, Frohnhöfer et al. 2016, Sing & Nüsslein-Volhard 2015, Fadeev et al. 2015, and White et al. 2008.

Although the adult pattern is fixed, zebrafish can modify the intensity of the dark stripes, making the skin pattern less conspicuous in lighter environments. Each melanophore contains hundreds of melanosomes (the organelles containing melanin), which have the ability to aggregate in the centre of the cell or disperse throughout the cytoplasm. The degree of pigment aggregation allows the animal to undergo rapid colour changes in contexts of mimetism, sexual and social interactions, and physiological stress (Pissios et al. 2006; Hutter et al. 2012). For instance, a zebrafish displaying behaviours of fear, such as freezing or erratic movements, quickly becomes pale, especially in light environments (Gerlai et al. 2000), whereas a zebrafish displaying aggression is generally darker (Gerlai et al. 2000; Larson et al. 2006).

ID methods

The simplest way to manage the problem of identifying individuals is to isolate each animal during an experiment. However, social deprivation can affect behaviour and development in gregarious animals (Hesse et al. 2015). In fishes living in groups, the social environment can affect antipredator behaviour, foraging and mate choice (Brown & Laland 2003), sexual and filial imprinting (Gómez-Laplaza & Gil-Carnicero 2008) and aggressivity (Halperin & Dunham 1993; Moretz et al. 2007). Furthermore, in a laboratory environment, shoal partners often represent the only source of environmental enrichment. Therefore, although individual identification is needed, it is optimal to avoid isolation of an animal. As such, various methods have been developed to address this need.

Visual methods

Direct observation of external morphology

Within a species, and particularly within a population or strain, zebrafish individuals tend to look similar, making identification a challenge (e.g. Sire et al. 2000; Cousin et al. 2012). Strains used in the laboratory tend to be populations with a high degree of in-breeding, with therefore weaker genetic variation, and thus potentially displaying more homogeneous phenotypic characteristics (morphology, physiology and behaviour) than in wild populations (i.e. Coe et al. 2008; Séguret et al. 2016). Nevertheless, a careful examination of the external anatomy (body shape, state of fins and opercula, detection of potential scars and unique colour patterns) can help this identification. These traits can be linked to individual development or related to trauma having occurred during the life of the individual (e.g. injured fins, loss of scales, evidence of regeneration, scars). However, for identification purposes, it is necessary to select characteristics that are maintained for a sufficiently long period, i.e. typically the entire life of the fish. Of course, the larger the group, the more difficult individual discrimination based on external body observation will be. The number of individuals maintained in groups is classically around twenty, but can go up to one hundred (Harper & Lawrence 2011). In the case of zebrafish patterns, some strains have high variations of skin patterns, whereas others show little to no obvious differences. Some strains lack skin patterns altogether. Generally however, when a skin pattern exists, minor variations can be sufficiently different between individuals to allow their individual identification. For instance, *choker* mutants display a colour pattern with obvious stripes but arbitrary orientation (Frohnhöfer et al. 2013; Volkening & Sandstede 2015) which can be unique enough to be efficiently recognizable (Fig. 3). Even with wild types, a careful inspection of stripes can allow the detection of small details specific to one individual (stripes showing small indentations or breaks, bridges between two stripes – see pictured examples in Fig. 4). Body pattern details can also differ between both flanks, thus increasing the possible number of discriminants, but thereby also making it necessary to take into account laterality when describing an individual.

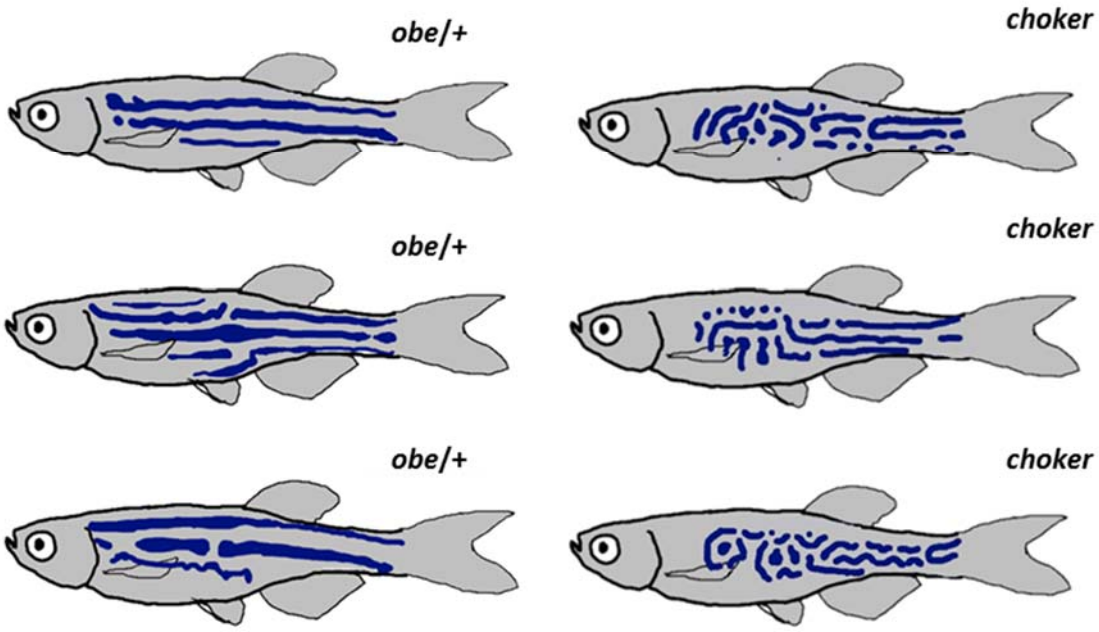


Fig. 3 Schematic drawing illustrating the possibility of identifying individuals based on the variations of the flanks' colour patterns (fin stripes are not illustrated). The pictures illustrate two zebrafish mutants where the phenomenon is very demonstrative: *choker*, a mutant with labyrinth patterns, and heterozygote *obelix*, a mutant with fewer stripes. Drawing inspired from pictures published in Volkening & Sandstede 2015 and Frohnhöffer et al. 2013 for *choker* individuals, and from Frohnhöfer et al. 2016, Maderspacher & Nüsslein-Volhard 2003 and Iwashita et al. 2006 for *obe/+* mutants.

The direct observation method requires the creation and maintenance of an up-to-date database of images (drawings or photographs) of one or both body sides, highlighting the main colour and shape differences. Adult patterns can be used for very long studies, as they are permanent unless an injury occurs. However, the more difficult it is to observe discriminant details, the longer the identification process will take. Directly identifying individuals inside a group is difficult, particularly in large groups: first, because finding an individual requires checking all fish in order to avoid false positives (Speed et al. 2007; Dala-Corte et al. 2016); and second, attempting to identify one target inside a group of distractors is difficult, as the presence of other similar fish moving in different directions can induce confusion in the human observer (Tosh & Ruxton 2006; Ioannou et al. 2008). It is possible to reliably distinguish individuals in groups of four or five (Reed & Jennings 2011) but, in larger groups, it is more convenient to briefly isolate an individual in order to check its colour patterns.

Several automatic "fingerprint" recognition systems based on external phenotype – software such as I³S (Van Tienhoven et al. 2007), StripeSpotter® (Lahiri et al. 2011), Identifrog® (Petrovska-Delacretaz et al. 2014), AmphIdent (Drechsler et al. 2015), ExtractCompare or Wild.ID (Bolger et al. 2012) – have been developed to identify individuals of species presenting variable natural marking patterns. These computer-assisted photographic identifications typically follow the same steps: first, a pattern is created based on a picture of the animal (some of these programs are even capable of identifying the 3D body shape and

orientation deformation); this pattern is then compared with a database of pre-existing patterns via software-specific algorithms (Sacchi et al. 2016). Finally, a classification of the best-ranked candidates is suggested to the user. To our knowledge, there are no published data on the success rate of automatic identification applied to zebrafish individuals.

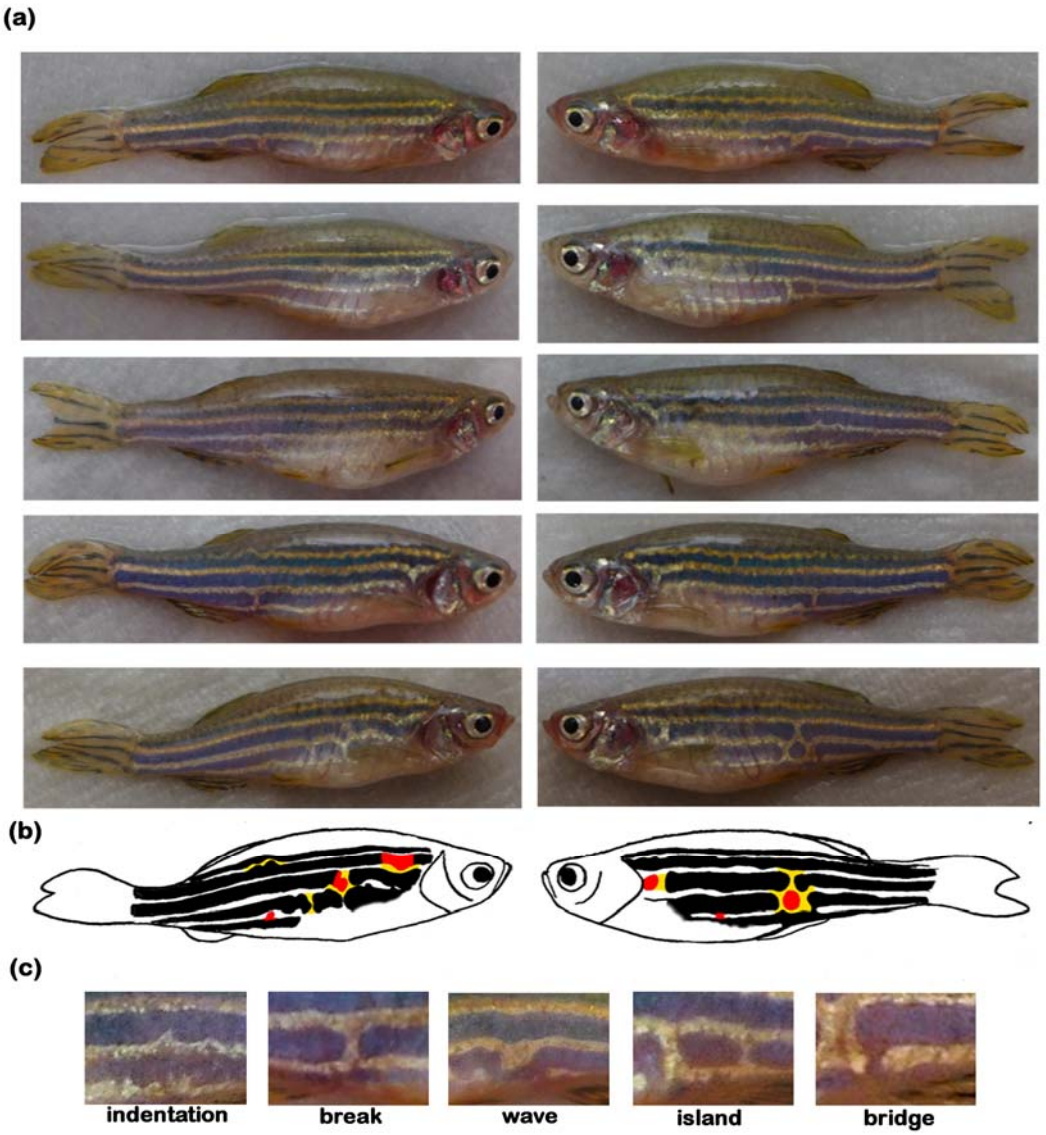


Fig. 4 (a) Examples of AB strain individuals with slight differences in skin pattern between individuals and body sides (side by side images show left and right flanks of one individual. (b) A drawing of the last individual of (a), highlighting some of the most obvious features (in red and yellow in online version) such as bridges, breaks and specific shapes, which make its identification possible. (c) Zoom on some distinguishing features.

Usually, visual identification remains possible when the fish changes shape (e.g. gravid female, scoliosis), as the reference database can be updated when necessary. However, visual identification is difficult or even impossible when, as is the case with certain strains, the mutants do not harbour any skin patterns (e.g. *casper* mutants, which are transparent due to the lack of chromatophores: Fig. 2). In such cases, individual identification at a distance (as is necessary

when observing behaviour in group contexts, in order to prevent any impact on fish behaviour) is very difficult and other methods must therefore be applied.

Fin clipping

Fin clipping – where a fin is partially removed to allow for subsequent identification – is probably one of the oldest methods for marking fish (Skalski et al. 2009). For instance, it has been frequently used to identify salmonids from breeding programs by snipping a section of the adipose fin (Saunders & Allen 1967; Hansen 1988). In zebrafish, fin clipping is more commonly used to sample tissues and DNA, and rarely to identify individuals. However, removal of a portion (1.5 mm) of the caudal fin has been used for zebrafish identification purposes by Saverino & Gerlai (2008) and Cheung et al. (2014). Although the method is quick and easy, there is still debate within the scientific community as to whether it may be harmful to fish. Some Institutional Animal Care and Use Committees (e.g. University of California, San Francisco) list several potential adverse effects of fin clipping in zebrafish. First, sectioned fins could negatively affect the fish's swimming performance (probably depending on the proportion of fin removed), and thus also its behaviour. Secondly, fin clipping could be a source of infection, which might also affect the fish's behaviour or possibly even its survival. While some studies suggest that fin clipping can reduce fish survival and growth (Saunders & Allen 1967; Hansen 1988) as well as cause suffering (Roques et al. 2010, Reed & Jennings 2011), numerous reports do not demonstrate evident deleterious effects (e.g. Champagne et al. 2008, Wagner et al. 2009). Furthermore, zebrafish are able to rapidly regenerate their fins: for instance, a zebrafish's quasi-completely sectioned caudal fin can be regenerated in around 3-4 weeks (e.g. Azevedo et al. 2011; Pfefferli & Jazwinska 2015). Such markings are therefore temporary and hence, for identification purposes, unreliable over time. Finally, the number of combinations of fin clipping is relatively limited, particularly on such small fish.

Scale regeneration method

Sire et al. (2000) have developed a method to recognize individuals based on the observation that the morphology of regenerated scales is distinct from that of normal scales, presenting a large regenerated focus devoid of ridges and grooves (Fig. 5B). The number and location of scales in zebrafish are constant and each scale can be identified by a matrix location system. They proposed the use of thirteen adjacent scales (numbered I to XIII from anterior to posterior) over four adjacent rows (A to D, dorsally to ventrally), on the anterior part of one flank, resulting in a total of 52 scales (Fig. 5). They suggested that the removal of four scales is sufficient to accurately identify an individual and that it is easier to remove a cluster or successive scales than four isolated scales (Fig. 5C). They recommend not using scales from the posterior part of the flank and from the caudal peduncle in order to avoid any possible impact on swimming performance, and because already-regenerated scales are more frequent in these areas.

To identify marked specimens using the scale regeneration method, the fish must be anesthetized and inspected using a stereoscope to determine which scales are regenerated. Such an inspection takes 1-2 min per fish (Sire et al. 2000). This method assumes a stability of scale pattern, without definitive loss of scales. However, other occurrences such as intra-specific fights, predator attacks, and hand or fishnet manipulation can involuntarily cause loss of scales, potentially making the identification difficult or even erroneous. Furthermore, as it is impossible to recognize regenerated scales without manipulating the fish under a stereomicroscope, this method is not suitable for behavioural observations.

Artificial colour methods

A variety of chemical markers can be used to mark fish (e.g. alcian blue, tetracycline, alizarin red, xylenol orange and calcein) by incorporation into mineralized tissues such as bones (notably otoliths), teeth and scales (Wright et al. 2002). Colour methods by incorporation are

not reported in post-larval zebrafish, although some have been successfully used on small juveniles or adult small fish such as killifish (*Heterandria formosa,* Poeciliidae) or guppies (*Poecilia reticulata,* Poeciliidae) (Leips et al. 2001; Bashey 2004). Some of the mentioned chemicals are fluorescent and can be seen optimally under UV light. This has the advantage of not affecting social behaviours or posing risk of predation under natural light. However, it must be noted that zebrafish are capable of perceiving UV light (Nava et al. 2011), and that the light emitted by fluorescence under UV is generally in the visible spectrum. These substances can be incorporated by subcutaneous injection (e.g. Meunier 1972, 1974; Meunier & Boivin 1974), by food ingestion, by immersion (Nagiec et al. 1988) or through osmotic shock, i.e. in a bath of saline water (Alcobendas et al. 1991; Mohler 2003). However, these methods are based on the diffusion of the chemicals into the body and are thus better adapted to marking and identifying a group rather than distinguishing between individuals. Moreover, these colours slowly fade with time, over the course of several weeks (Leips et al. 2001; Bashey 2004). Last, some of the chemicals need to be administered in low concentrations due to their toxicity. For instance, tetracycline is an antibiotic which, at high concentrations, shows an effect on survival, growth and behaviour (Zhang et al. 2015a, b), as well as presenting an environmental risk (Bashey 2004).

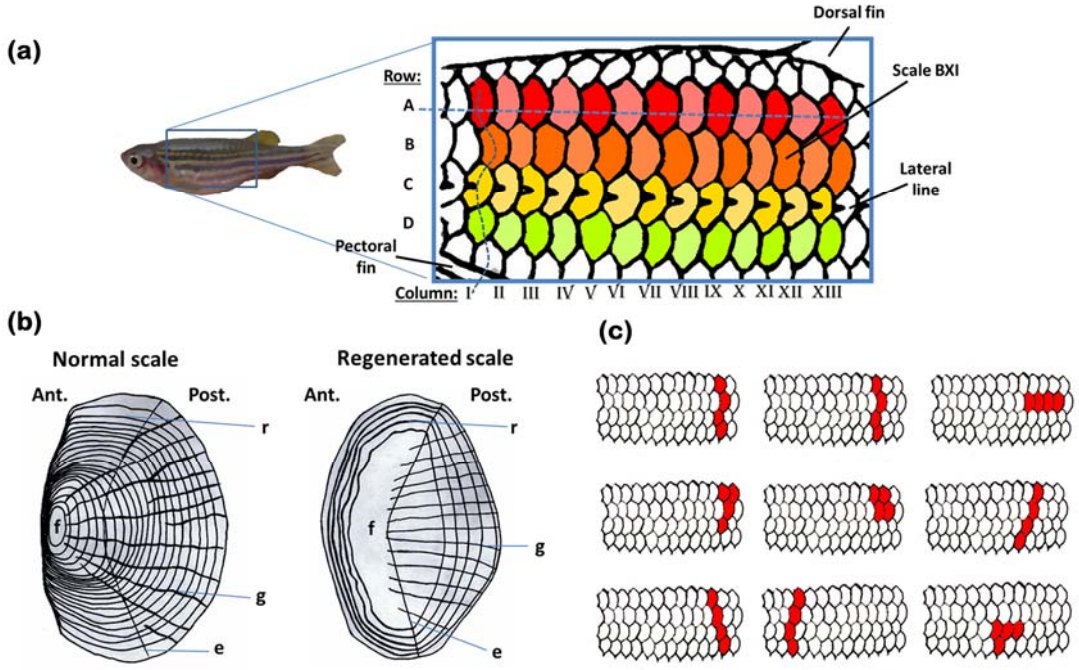


Fig. 5 Sire's scale regeneration method. (a) Matrix definition to identify a scale within the rows A to D, and columns I to XIII. Colours (gray levels in the paper version) are used to help the lecture of the matrix and do not correspond to any colour tagging. (b) Compared morphologies of a normal and a regenerated scale in adult zebrafish (f: focus, r: ridges or circuli, g: grooves or radii, e: edge of epithelium cover, which covers the scale on its posterior part, Ant.: anterior, Post.: posterior). (c) Some examples of ID codes (identifier scales are highlighted dark red).

Intradermic colour tattoos (by needles or dermojet, i.e. high pressure needle-free injector) are a common identification method used on many fish but have not been widely tested on zebrafish. Cheung et al. (2014) evaluated tagging using dyes developed specifically to stain tissue (i.e. Sigma-Aldrich MDT-100-1KT available in five colours: red, yellow, green, blue and black). They marked zebrafish on the rostral part of the caudal peduncle, injecting ink at a depth

of 2 mm subcutaneously, between the skin and muscle. All available colours are clearly visible on wild-type zebrafish, although green and blue are harder to distinguish due to the species' natural colouring. Both the size of the coloured area and colour saturation decreased over the weeks following the injection, but marks were still observable 1 month post-injection. Visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Shaw Island, WA, USA; Fig. 6) are available in ten colours (six are fluorescent to UV light). They are stable in time and, injected into a transparent body area, allows external individual identification. Tag visibility depends on skin pigmentation, depth of injection and the quantity injected. VIE tags have been used in behavioural studies, both for manual (Bolliet & Labonne, 2008; Bolliet et al. 2007) and automatic recording of activity using a video multi-tracking system (Delcourt et al. 2011, 2013). Hohn & Petrie-Hanson (2013) have shown that these VIE tags modify neither mortality nor growth in juvenile and adult zebrafish. Even individuals as young as 1 month post-hatching (i.e. around 90 mg and 2.25 cm long) can successfully be tagged with VIE without affecting survival. Hohn & Petrie-Hanson (2013) found that tagging the dorsal fin base with VIE tag (2 mm strip) provided a full 1-year retention rate, whereas tagging other body parts caused tag losses. They have also shown that the pink and red VIE tags are the most detectable, followed by yellow and orange. However, it is difficult to distinguish between yellow and orange tags, and between pink and red tags. Thus, although VIE tags are an interesting method to tag fish for laboratory management, the relatively small tag size and tag location in zebrafish makes it difficult to ID at a distance, either by the human eye or automatically by video tracking systems (Miller & Gerlai 2007).

Fig. 6 Example of colour tagging on zebrafish using VIE tag (red elastomer observed here in natural light, picture is slightly saturated for illustration – whitish line in paper version). This individual was tagged 3 years before the picture was taken, at the age of 16 months, underlining that a tag can persist for a very long time, even if a part of it has been rejected (initially, the tag was a continuous line).

Transgenic tools

Natural fluorescence is widespread in fish, exhibited by more than 180 species in 50 families, essentially in marine species and particularly in cryptic species living in coral environments (Sparks et al. 2014). Although zebrafish do not naturally have this ability, transgenic zebrafish have been engineered to express fluorescent proteins through the expression of cnidarian DNA inserted into the zebrafish genome (Wan et al. 2002; Gong et al. 2003). Classically, in many transgenic strains, the expression of fluorescent proteins is used to label specific cell or tissue types (Harper & Lawrence 2011). In the GloFish® strain, fluorescent proteins are expressed in skin and muscular tissues, and are externally visible under natural light and magnified under UV light.

To date, six fluorescent colours have been commercially produced: red, green, pink, purple, blue, and orange. Although the number of colours is currently limited, their combinations – through simultaneous expression of several different colours – open a very large range of possibilities. For instance, in neural studies, the "brainbow" method makes it possible to distinguish a large number of neurons using fluorescent proteins (Hampel et al. 2011). By randomly expressing different ratios (through multiple copies of each transgene in different proportions) of red, green, and blue fluorescent proteins in each neuron, it is possible to label each nerve cell with a distinctive hue (Livet et al. 2007; Hampel et al. 2011). The technique works in the same way as a television uses the three primary colours to generate all colour tones. The palette of combinations obtained results in nearly one hundred different colours. A similar system, where each individual fish would have its own colour (or its own body pattern of one or several colours), could allow rapid visual identification of individuals. The brainbow method has already been developed in zebrafish skin to study epithelium regeneration (Skinbow in Chen et al. 2016).

However, the expression of a new gene can potentially induce physiological and behavioural effects in the transgenic individual, which could then also modify its social interactions and fitness. Transgenic fluorescent zebrafish have a lower fitness (lower fecundity, fertility, hatching rate and slower growth) than wild-type zebrafish (Khee 2006; Nagare et al. 2009; Hill et al. 2011). Impacts were also found on mating preferences, with female zebrafish preferring wild-types males over green males (Hill et al. 2011) but preferring red males over wild-type males (Owen et al. 2012). Although the white cloud mountain minnow *Tanichthys albonubes* (Cyprinidae) shows a stronger shoal preference for transgenic red over wild-type individuals (Jiang et al. 2011), no such preferences were found in zebrafish with respect to red and wild-type zebrafish (Snekser et al. 2006). As each transgenic fluorescent type can potentially differ physiologically and behaviourally from each other as well as from the wild types, using a mixed group of transgenic individuals in a study may lead to uncertain results due to the heterogeneity of the group and of their inter-individual interactions. We consider here only the use of transgenic individuals in laboratories without considering the risks for the environment and natural populations (for a review of these risks, see Maclean & Laight 2000; Muir, 2004; Prahash et al. 2011; Devlin et al. 2015).

Electronic methods

In the last decades, the use of electronic tags has revolutionized behavioural ecology, as these systems make it possible to obtain a large quantity of data, both in the field and the laboratory, by continuously recording activity, movement, environmental conditions and even physiological parameters of the fish (Lucas & Baras 2001; Cooke et al. 2012; Thorstad et al. 2013). These electronic tags can be external (Jepsen et al. 2005) or internal, implanted either surgically inside a body cavity (Cooke et al. 2011) or by gastric insertion via the mouth (Thorstad et al. 2013).

PIT tags (RFID)

In the laboratory, the most commonly used tag is the Passive Integrated Transponder (PIT) (e.g. Winandy & Denoël 2011; Cousin et al. 2012), a RFID system. A PIT-tag structure consists of a ferrite-cored antenna coil and an integrated circuit, both encapsulated into biocompatible glass in order to protect the electronic components and prevent tissue irritation. This tag is "passive" as it does not carry a battery. It can be activated via an external energy source, in this case a magnetic field, which causes the PIT-tag to send an ID code to a reader, allowing identification of the individual. In practice, the reader generates a continuous magnetic field, providing the tag with the energy needed to emit the ID code; therefore, such tags have an essentially unlimited life expectancy.

Low Frequency (LF) FDX (Full-duplex) PIT-tags work usually at a frequency of 134.2 KHz and are of suitable size to mark zebrafish: a diameter of 7-8 mm, a length of 1.25-1.35 mm and a weight of 30 mg (Fig. 7). These products are commercialized as being adapted for use on zebrafish, but no study concerning their potential impact on zebrafish behaviour and welfare has been published. Major limiting factors of PIT tags are their size, their weight and the size of the needle used for insertion. Nowadays, most researchers accept the "2%-rule", which states that the tag should not exceed 2% of the fish's weight. For zebrafish, sexual maturity is reached at 3 months and optimal breeding capacity is maintained until 18 months of age, after which it decreases with age (Avdesh et al. 2012). The period between 3-18 months of age is thus typical for studying zebrafish, even if they can reach 4-6 years in the laboratory (Gerhard et al. 2002). Males and females can reach a mean weight of 0.3-0.4 g, respectively at sexual maturity, and about 0.6 or 0.8-1 g, respectively at the age of 1.5 years, when their growth becomes very limited (Ribas & Piferrer 2014). While the "2%-rule" is more a guideline than a strict rule, some authors consider it too strict (Winter 1996; Jepsen et al. 2005; Smircich & Kelly 2014). As a consequence, it would have to be raised to 5% for the LF FDX tags to be useable in sexually mature zebrafish.

Another glass-encapsulated FDX PIT tag, working at higher frequencies (13.56 MHz) and currently the smallest on the market with a length of 6 mm, a diameter of 1 mm and a weight of 10 mg (Fig. 7; Nonatec RFID, Lutronic International, Rodange, Luxembourg), was successfully used on zebrafish (Cousin et al. 2012; J. Delcourt, pers. obs.). This tag is three times lighter than the smallest LF PIT tags. Considering the "2%-rule", it can be used on fish with a minimum weight of 500 mg. However, Cousin et al. (2012) have successfully tagged juveniles of around 350-450 mg (representing 2.8% of body weight), observing no impact on growth and mortality rate, and no lasting effect on behaviour up to 2 months post-tagging. However, Cousin et al. (2012) observed that after 5.5 months, 10.7% of zebrafish tags cannot be read (without distinction between rejections and defective microtags).

Finally, a new RFID using UHF (Ultra High Frequencies, around 900 MHz) has been used to tag very small insects such as ants and vinegar flies (http://www.rfidjournal.com/articles/view?12592 – SK Electronics Co, LTD Japan). Measuring 0.46 × 0.48 mm, this RFID is currently the smallest ever used for studying animal behaviour. However, its reading range is limited to 2 mm.

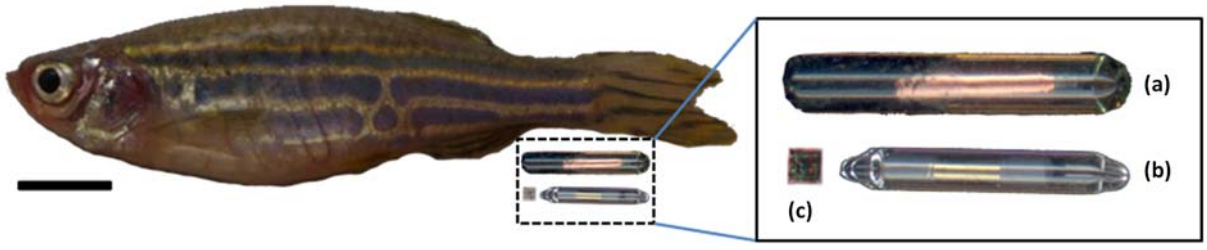


Fig. 7 Female AB zebrafish, 38mm long and weighing 880 mg. The scale bar represents 5 mm. Three electronic tagging methods suggested for use on small fish are shown under the fish, both to scale and magnified (inset): (a) LF PIT-tag 8 mm (± 30 mg), (b) HF PIT-tag 6 mm (10 mg) and (c) p-Chips from Pharmaseq (length: 0.55 mm; 82 µg). LF: Low Frequency, HF: High frequency, PIT: Passive Integrative Transponder.

p-Chips tags

As an alternative to RFID tags, the very small p-Chips® tag (PharmaSeq, Inc., NJ), with a size of 500 × 500 × 100 µm and a weight of 82 µg, is the smallest electronic animal tag on the market. p-Chips have already been used to tag mice (Gruda et al. 2010), smaller animals such as ants (Robinson & Mandecki 2011; Robinson et al. 2009, 2014) and honey bees (Tenczar et al. 2014), or even as insect pins in zoological collections (Jolley-Rogers et al. 2012). This tag is composed of photocells which, when excited by pulsed laser light (658 nm wavelength), provide power and synchronization signals for electronic circuits, which then transmit an ID to a reader. The 30-bit memory capacity allows for more than 1.1 billion unique IDs. The chips are stable, chemically inert, resilient to physical extremes and biocompatible.

p-Chips have been used to successfully tag zebrafish. Chen et al. (2013) obtained 96 % of retention after 16 weeks, and mentioned no difficulty in reading the tags. The p-Chip was inserted into the trunk's dorsal muscle using a specialised injector (Gruda et al. 2010). Chen et al. (2013) found no obvious impact on the animals' health, but quantitative evaluation is still missing. This system seems thus very useful for tagging zebrafish or other small fishes for laboratory management. The identification process requires removing the fish from the tank and sedating it, locating the p-Chip's position, and scanning it with the ID reader. Unfortunately, the short reading range of 4 mm disqualifies this system from identification at a distance, and thus from use in socio-behavioural studies.

Table 1 summarizes the technical details of micro-electronic tags available to tag small fish and their adequacy for zebrafish.

Table 1 Summary of small micro-electronic tags available to tag small fish and their adequacy for zebrafish

Electronic system	Energy source	Size and weight	Memory	Read distance	Target animals	For zebrafish?
FDX RFID PIT-tag	LF (134.2kHZ)	8 × 1.25 - 1.35 mm (~30 mg); Needle[a]: ≤ 1.65 mm	Read-only or read-write	< 10 cm	Relatively small fish (> 1.5 g[b])	possibly (not tested yet)
FDX RFID PIT-tag (Nonatec)	HF (13.56MHz)	6 × 1 mm (10mg) Needle[a]: 1.27mm	512 bits (can be read and rewritten)	~12 mm (in air)	Small fish	Validated (Cousin et al. 2012)
FDX RFID PIT-tag (SK-electronics)	UHF (900MHz)	0.46 x 0.48 (× ±0.1) mm Needle[a]: 0.82mm	512 bits	< 2 mm	Ants, fruit flies	Not yet tested (waterproofing to solve)
p-chips (Pharmaseq)	Laser (658nm)	0.5 × 0.5 × 0.1 mm (82µg) Needle[a]: 0.82mm	30 bits	< 5 mm	Ants, bees, mice, small fish	Validated (Chen et al. 2013)

Comments: [a]nominal outer diameter; [b]theoretically based on the "2% of body weight" rule of thumb". LF, HF and UHF: Low frequency, high frequency, ultra-high frequency; FDX: Full-duplex. In read-only tags, stocked information cannot be modified; in read-write tags, the tag can record new additional information.

ID during video multi-tracking

The recent development of digital imaging techniques provides new opportunities to study and track individual fish inside social groups such as shoals and schools (Delcourt et al. 2009, 2011, 2013; Delcourt and Poncin 2012), including small species such as zebrafish (Kato et al. 2004; Mirat et al. 2013; Pérez-Escudero et al. 2014). Video tracking consists of tracking moving objects (in this case, individual fish) and monitoring their activities via image sequences obtained from video cameras (Maggio and Cavallaro 2011; Delcourt et al. 2013). An automated procedure determines an animal's position over time and delivers the resulting tracks as well as a large array of data such as distance travelled, speed or space used (Noldus et al. 2001; Maggio and Cavallaro 2011; Denoël et al. 2013). Video multi-tracking allows several individuals to be tracked simultaneously within the same water volume, thus integrating the interactive social component into animal behaviour studies.

With multi-tracking, individuals must be identified over time. Different techniques have been developed to reach this objective. Several are based on morphological differences between individuals (e.g. different sizes [Hansen et al. 2008] or colour phenotypes [Ylieff and Poncin 2003]) or on colour tagging (Delcourt et al. 2011, 2013), making it possible to track a very small group of fish. Tracking zebrafish is very challenging because individuals are phenotypically very similar. Automatic detection of coloured tags, as suggested by Cheung et al. (2014), has never been tested on this species. Recently, several methods have been developed which allow the tracking of unmarked individuals. Typically, these methods work by alternating two analysis phases: an observational phase followed by a predictive phase (Delcourt et al. 2013). The observational phase consists of detecting each individual as a tracked target and obtaining its location. Detection is possible if the fish's image has a detectable characteristic (e.g. if it is darker than the background). The minimum position information is the coordinates of a single point (e.g. the centre of mass of the fish's image, or the tip of the nose; see Delcourt and Poncin 2012). More complex information, including body orientation and the position of different body parts such as the tail and the head, requires a careful analysis of the body shape by specific image software. In any case, detecting the position of a fish at a given time is not sufficient to determine its identity. The second phase of the analysis is therefore to connect the successive positions of each individual over time in order to obtain its trajectory. Generally, the predictive part is critical due to the risk of misidentification (Delcourt et al. 2006, 2009, 2013).

Identification errors frequently happen when the fish images are occluded (e.g. when two or more fish images become superimposed during a period). To solve these problems, several strategies have been tested. One is to use statistical prediction of future individual positions, based on their previous short-term motion as well as on the species' typical motion behaviour (see Delcourt et al. 2013). Used successfully on some invertebrates, these predictive methods have been less developed in fish tracking. Another approach is based on measurement of the tri-dimensional coordinates (by stereo-cinematographic vision [Viscido et al. 2004; Hemelrijk et al. 2010; Maaswinkel et al. 2013]. or by detection of multiple shadow projections using several lamps [Laurel et al. 2005]), significantly limiting occlusion problems. However, major recent advances in fish multi-tracking, generally tested on zebrafish groups, have come from image analysis of the fish themselves (Fig. 8). Kato et al. (2004) suggested an image treatment known as erosion-dilatation, which allows the separation of low-resolution blobs into well-defined islands of pixels corresponding to each fish. Also for use on low-resolution images, Dolado et al. (2015) have used an ellipse technique to ID the fish locations. Another approach is through high-resolution body shape analysis, generally with high frame rates. Typically, a model of the fish's body shape is used to fit and thus detect each fish. The challenge of these models is to manage the complex, highly variable and flexible shape of fish. Butail and Paley (2010) have developed a similar system in 3D by using two cameras. The shape model,

which is the result of the three-dimensional form projected onto a plane, takes into account the bending of the body. Other models have been applied without the need to use 3D, simply by filming from above. In the continuity of work by Fontaine et al. (2008) and Mirat et al. (2013), Qian et al. (2014) proposed a system able to detect the head of the zebrafish first, based on fitting an ellipse, with the head being considered as a rigid part, and the tail as a bend. Wang et al. (2016) suggested another method, through analysing boundary curvature of the body to detect the location of the tail and the head, as these two parts have a strong change rate in their boundary curvature (fins are not visible in the image treatment). This analysis also works in the case of occlusion (Fig. 8). A body model that uses a chain of rectangles of different sizes is then applied to fit the fish body.

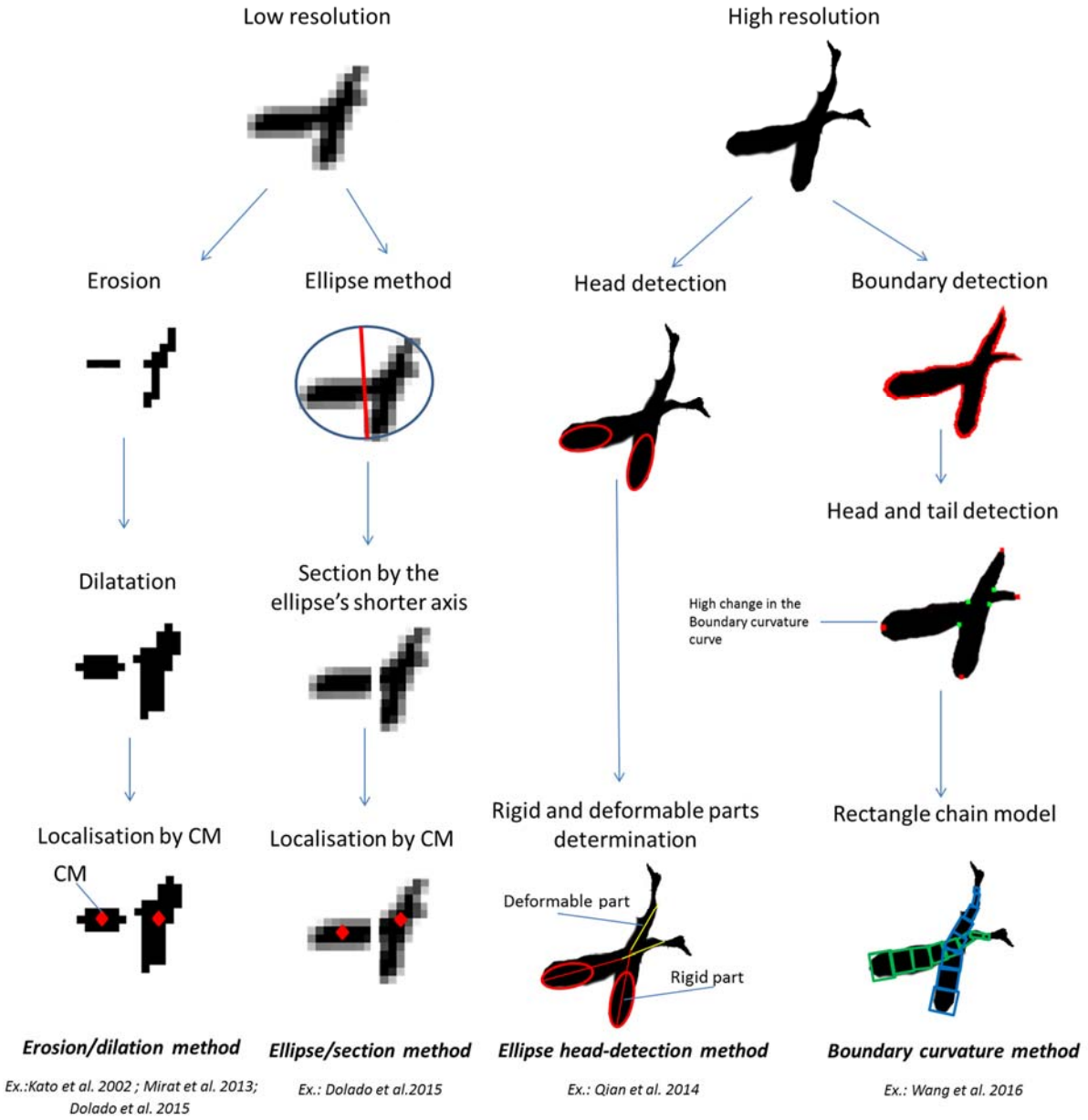


Fig. 8 Various methods for resolving individual identification during occlusion in zebrafish research using video multi-tracking systems, provided that the fish's identity is known before the occlusion. ◊ and CM: centre of mass of detected pixel island, attributed as the coordinates of the fish.

Although multi-tracking allows one to follow all individuals during an entire video sequence, the individual identification of each fish remains unknown between two non-continuous videos. Pérez-Escudaro et al. (2014) have proposed a "fingerprinting-based" tracking system and tested their system (IdTracker) on a variety of animal species, including zebrafish. The method is based on the comparison of intensity of each pixel i_p of the detected individual with each other pixel of the same animal, coupled with the distance d between each duo of pixels. This method creates a histogram (i_1+i_2, d) with a unique pattern, which can be used to discriminate between individuals on the screen, image after image. This system is capable of detecting and identifying each individual between different video sequences, even if the videos were recorded several weeks apart. However, a unique fingerprint is probably less efficient at recognizing the same individual over several weeks if the body shape changes, as is the case, for example, with a gravid female. During occlusion, the method is not able to identify overlapping individuals; another algorithm is used to estimate the position of individuals based on their location before and after the occlusion.

Despite significant improvements, the perfect multi-tracking system for zebrafish without identification errors is not yet available. As misidentification errors are essentially associated with occlusion events, the larger the group size or individual density, the greater the number of errors likely to occur. Generally, the performance of these recent systems at correctly tracking an individual during several thousand frames is superior to 99% when the group size is moderate (around ten), but can rapidly decrease for larger groups (Wang et al. 2016).

Cross-comparisons of ID methods

Detection range

Detection range is defined as the maximum distance between the ID signal and the receptor (the observer, the camera or the antenna). This distance depends notably on the size of discriminant characteristics and the ease of distinguishing similar marks. A long detection range is very important for behavioural studies requiring identification of fish while they are swimming freely, where handling or even experimenter proximity must be avoided.

Scales or fin clipping are not easy to observe without handling the fish. This is also the case for microchips (e.g. p-chips) and HF PIT tags, which have very short detection ranges (respectively < 5 mm and 12 mm in air), making it necessary to handle the zebrafish in order to read the ID code. Skin patterns generally require very close observation. LF PIT tags allow detection of fish passing through or near a fixed antenna (Ovidio et al. 2017) or by approaching a mobile submersible antenna to the tagged individual (Winandy et al. 2017). Their detection range is proportional to the tag size. For instance, 8 mm and 12 mm LF PIT tags have a reading range of respectively 8-10 cm for the former (Ousterhout and Semlitsch 2014), and greater than 30 cm for the latter (e.g. Cucherousset et al. 2005). Depending on the spot size and colour combinations, colour tags can be detected from a greater distance (e.g. around 1 m in dim natural light for fluorescent VIE tags enhanced under UV light).

Lifespan and retention rate

The lifespan and retention rate of tagging methods are a crucial point to consider. Typically, the method must be efficient and reliable during the full duration of its use. If its lifespan is too short, re-tagging the fish can induce more stress, potentially causing deeper injuries and badly healing wounds, especially if the tagging location is the same. A technique lasting the fish's

entire lifespan is ideal. However, many methods have a limited working duration, with the tag being rejected or attenuated over time.

Typically, tattoos and fin clips remain visible for several weeks only, whereas the scale method can be used for several months if no alteration or scale loss occurs. VIE tag loss occurs very frequently in zebrafish except for very thin tags located on the dorsal part (Hohn & Petrie-Hanson 2013). Electronic methods allow the identification of individuals during their entire lifespan. However, electronic tags can sometimes be rejected, even several months after tagging (J. Delcourt, pers. obs.). Concerning fluorescent transgenic zebrafish, as the fluorescent proteins are produced continuously throughout the animal's life, identification is possible during its entire life.

Rapidity and repetitiveness

Overall, an advantage of tags is the possibility of frequent identification of each individual. Some methods are less adapted to frequent use. For example, the scale method implies removing and replacing scales, and can only be carried out a limited number of times. Visual tags are easy to re-read if they are stable in time (no fading) and easy to distinguish from the fish's natural skin colour. The electronic tags can be reread infinitely, and the tag identification can be transferred directly to a computer, thus limiting human errors.

Another important point, particularly when identifying a large number of fish, is the speed of identification. In zebrafish, as visual techniques (e.g. natural skin patterns, fin clipping, scale method, colour dyes) are still based on direct observation by natural vision, it can take a significant amount of time to identify individuals within a large group, particularly if the distinguishing features are difficult to detect. With electronic methods, identification can be very quick (e.g. up to ~70 HF PIT-tags/min).

Maximum number of ID codes

If individual identification is required, the number of ID codes determines the size of a studied group. This number varies widely depending on the ID method. To our knowledge, mixing individuals tagged with different methods, or cumulating different methods on the same individuals, have not yet been explored. Methods adapted to tagging a group allow only a binary choice (either from the marked group, or not) and are not adapted to individual identification (e.g. colour incorporation).

Although fluorescent transgenic zebrafish are limited to six available colours, developing multi-coloured individuals could greatly increase the number of possibilities. Individual identification with multiple fin clipping locations is possible, but the number of possible combinations is limited. For instance, using four caudal fin incision locations, only 15 combinations can be obtained. Using a combination of differently clipped shapes can increase the number of combinations. The use of colour tags also gives a relatively limited number of combinations, but both flanks can be used. In the Cheung et al. (2014) method, the number of combinations is 24 when using five of the six available colours, and two potential locations per flank. Of course, increasing the number of locations or colours substantially increases the number of combinations. Visual methods based on skin patterns are generally limited to a few dozen individuals, depending mostly on the ease of detection of individual characteristics. The scale method, using one flank, can allow for 270,725 combinations of 4 scales removed from 52 potential choices. However, as Sire et al. (2000) advise the use of contiguous scales, for which the number of possibilities then diminishes to a couple hundred combinations. In the case of electronic methods, the number of combinations is virtually unlimited.

Minimum size and age

The choice of the tagging method is largely determined by the relative size of the tag compared to the fish. Therefore, the number of methods is more limited for zebrafish than for larger species. With the colour methods, the quantity of marker injected determines the size of the tag.

Cousin et al. (2012) have successfully tagged juvenile zebrafish as small as 350 mg with HF PIT tags, even one individual of only 178 mg, but without thoroughly estimating potential behavioural impacts. LF PIT tags (8 mm) work well for individuals more than 600 mg, whereas the suitability for use with smaller fish remains to be tested (J. Delcourt, pers. obs.). According to PharmaSeq, p-Chips could be used to tag juveniles from 20 mm long (i.e. around 200 mg, weight estimation based on Ribas & Piferrer 2013). With VIE tags, Hohn & Petrie-Hanson (2013) tagged 1-month-old zebrafish weighing 90 mg. For embryos and larvae, their translucence allows the observation under UV light of colour incorporated into bone. However, this technique is better adapted to the tagging of populations than to individual identification. Last, the scale, fin clipping, and skin pattern methods require the respective morphological structures to be well developed (from the late juvenile stage onwards), and therefore cannot be used on younger individuals.

Impact of tagging and ethical considerations

The crucial point of studies using ID markers is the necessity to not alter the viability, growth, fitness or behavioural traits of tagged individuals (e.g. Thorsteinsson 2002). These welfare considerations are not limited to the impact of tagging itself but extended to the entire experimental procedure where each step (catching, handling, tagging and recovery) can have an impact. Choosing the optimal tagging method and procedure is not only essential to respecting the ethical standards for the use of experimental animals, but is also required for successful breeding and even research, as altered physiology and behaviour could lead to serious flaws in the study (Roques et al. 2010; Fürtbauer et al. 2015; Sneddon et al. 2016). In laboratory management, decreased welfare will have a direct negative impact on the success of maintenance, growth and reproduction of laboratory populations (Schreck et al. 2001; Castronova et al. 2011). In nature, modifications of appearance or behaviour can increase predation risks because the fish is easier to detect and/or capture (Jepsen et al. 2015).

Biocompatibility requires the tag to be stable, and to not cause any injuries or discomfort to the animal, as well as not to be toxic nor affect physiological processes. For instance, electronic tags are typically encased in a glass capsule with no abrasive surfaces. In colour methods, some chemicals are stable (e.g. VIE), whereas others are toxic when used above specific, very low concentrations (e.g. tetracycline).

If the tag is too heavy or large, negative effects can be expected on the animal's behaviour and even health. In this case, the tagged individual may endure significant difficulties in moving, suffer a potential modification to its hydrodynamic profile, experience a limited degree of movement, and possibly, bear pressure on its organs (Winter 1996; Jepsen et al. 2005; Smircich & Kelly 2014). The "2%-rule of thumb" previously discussed depends on the species and life stage but could be extended to 5% for zebrafish (Cousin et al. 2002; J. Delcourt, pers. obs.).

In social species like zebrafish, tagging an individual can modify its morphology or behaviour, and consequently, may potentially impact the behaviour of others, such as sexual or shoal partner preferences, or aggressive interactions (Owen et al. 2012; Frommen et al. 2015). No social impact has yet been reported in methods which are presumed to be undetectable to shoal partners, such as electronic methods, removed scales, or use of colour only detectable under artificial light.

Applications to other small fish species

The ID methodologies presented here to study zebrafish can also be applied to other similar-sized fish that could therefore equally benefit from similar ID methods, both in the field and in the laboratory (see also Skalski et al. 2009). However, the relevance of ID methodologies to other species depends on their morphological and behavioural characteristics.

Although less commonly used, numerous other fish species can be recognized individually by their natural marks and patterns – on the condition that these characteristics are sufficiently polymorphic and rich in information content to be able to discriminate one individual from the others, and that they remain consistent over time (i.e. Correia et al. 2014). Though the use of stripes is specific to zebrafish, numerous other skin patterns and colours, as well as bony structures (Freret-Meurer et al. 2013; Dala Corte et al. 2016) found in other fish species can also allow the photo identification method.

Few tagging methods were reported on species of similar size to the zebrafish. The most frequently used ID tagging method is the VIE, which is applied, for instance, in guppies *Poecilia reticulata* (Croft et al. 2004; Reznick & Bryant, 2007; Piyapong et al. 2010), mosquito fish *Gambusia holbrooki* (Poeciliidae) (Chapman & Warburton, 2006), glass eels *Anguilla anguilla* (Anguillidae) (Delcourt et al. 2011), and medaka *Oryzias dancena* (Adrianichthyidae) (Im et al. 2017). The choice of the most visible colours varies depending on the natural pigmentation of each species (Curtis 2006). In the case of juveniles, the visibility of VIE tag can decrease with the growth of surrounding tissue and modification of pigmentation (Frederick 1997). PIT-tagging has been used to ID a large variety of fish species, including moderately small individuals (Cucherousset et al. 2005), but was rarely applied to fish of weight and size like zebrafish (e.g. > 55 mm with PIT-tag in salmon: Prenctice et al. 1990). Fin clipping and scale regeneration methods could also be used on a variety of species.

Video multi-tracking methods could also work on various fish species (Baatrup 2009; Tunstrøm et al. 2013), particularly in single tracking mode. However, low contrast or translucent species will make this technique challenging without using colour marks (Delcourt et al. 2011).

Choice of best ID method and potential applications

Four general situations of ID control can be encountered in the laboratory (Suppl. Fig. 1). The first situation is in stock management where identifying individuals is often necessary at regular time intervals and in different storage aquariums. This can occur during the importation and exportation of individuals from and to other laboratories or institutions, during the transfer and redistribution of fish to new storage tanks, during routine checking (e.g. health status or body measures), as well as for the identification of a dead individual.

The second situation is in the control of genitors for the production of hatchlings that are frequently used in developmental studies, drug screening and toxicological research (Ahmad et al. 2012; Kalueff et al. 2013). In this context, the identification of genitors is required for better control of individual variability in an experimental group, and for the transgenerational transfer of genotypes or phenotypes (Ho & Burggren 2012). The parents' identities can also be required in studies on heritability (Ariyomo et al. 2013), sexual determinism (Liew and Orbán 2014) and in the production of stable transgenic or mutant strains through mating clearly identified carrier parents (Higashijima 2008; Harper & Lawrence 2011). Avoiding reproduction between relatives is also important in this context.

Third, individual identification is useful in experimental programs with repetitive trials during which individuals are tested on several occasions. Typically, the aim of replicated

studies using the same individuals is to determine changes over time, such as those which can arise due to age and size increase, learning and habituation processes modifying the individual responses, cumulative effects of treatments, or even the recovery period following a treatment. Repetitive trials also enable an increase in the statistical power and help avoid the use of a large number of individuals (e.g. Papoulis & Pillai 2002).

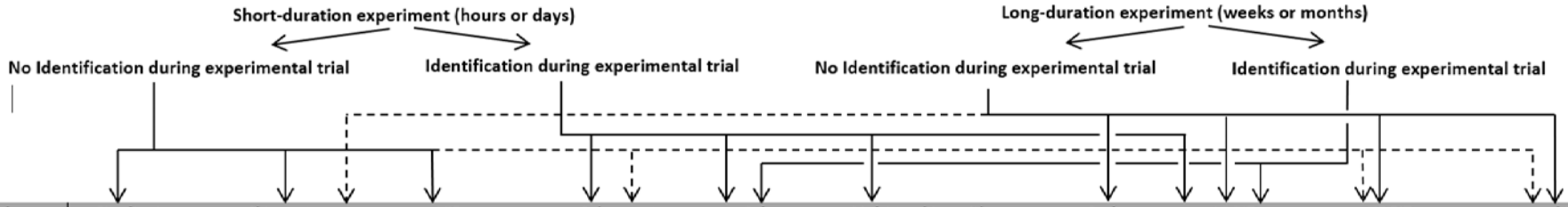
Table 2 Specifications and available ID methods depending on the four main contexts of zebrafish use in the laboratory

Specifications	Suggested ID methods
Stock management :	
- long period (months to years) - large number of ID codes	- LF PIT tags, HF PIT tags, p-chips, skin patterns, scale methods[a]
ID of reproductive parents:	
- long duration (months to years) - moderate to large number of ID codes - no impact on mating behaviours & reproductive capacities	- LF PIT tags[b], HF PIT tags[b], p-chips, skin patterns, scale methods[a]
Experiments with repetitive trials:	
- moderate to large number of ID codes - avoiding methods requiring long examination and/or systematic use of anesthesia - duration depending of the duration experiments	*Short-duration experiments (days or few weeks):* - Colour tattoo, VIE tags, fin clipping[c], skin patterns *Long-duration experiments (months or more):* - LF PIT tags, HF PIT tags, p-chips, skin patterns, scale methods[a]
Experiments with individuals in groups:	
- low to moderate number of ID code - live monitoring of identity - long reading range - absence of any effect of tags or marks on the behavioural patterns of tagged and the others	- LF PIT tags[d], Colour tags[e] (dye, VIE, tattoo), coloured transgenic fish, video multitracking

Comments: [a] if no loss of scales occurs during the use period; [b] intraperitoneal insertion, which can potentially have an effect on reproduction; [c] number of combination very weak; [d] Currently, impossible to read simultaneously two or more tags in the same time by the same antenna, if fish are too close, under-detection is frequent; [e] only if colour tags have not impact on behaviours of the tagged fish and others group partners. Some methods can only be applied to larger individuals (e.g. LF PIT tags).

Fourth, the last context is specific to behavioural studies, where individual identification is necessary to determine the location or behavioural patterns of each individual over periods of time (usually spanning from minutes to hours) in a group. This involves several zebrafish being transferred together to an observation aquarium, which can have a specific setup such as a labyrinth or a T-maze. There are two typical ways to use these ID methods. In the first, ID readers are placed at a specific location (often at the transition between different experimental zones, such as at the entrance of a response chamber); typically, the recorded data is limited to the ID number and the detection time, which are associated to already known positions. By including information on the individual's direction, this system can determine at any time in which zone of an arena the individual is located, even without knowing its precise coordinates. This type of experimental setup could help in social studies, in particular those concerning collective decision-making and stability of leadership (Miller et al. 2013). The second way is stricter, requiring continuous tracking of each individual's identity and precise location at all times. Tangible examples of multi-tracking of zebrafish or other small fishes are the study of pathological social behaviours (Stewart et al. 2014a,b), social networks (Croft et al. 2004; Krause et al. 2015), or collective motion (Miller & Gerlai 2012; Tunstrøm et al. 2013; Qian et al. 2016).

Table 3 Synthesis of the advantages and disadvantages of methods which can be used to identify individual zebrafish, with a simplified decision tree for the selection of the most adequate method.



Methods	Fin clipping	Skin pattern	VIE tag	Colour tattoo	Colour transgenic	Multi-tracking without tagging	Scale regeneration	LF PIT tags	HF PIT-tags	p-chips
Type of methods	Visual	Visual	Visual (colour)	Visual (colour)	Visual (colour)	Visual (computer analysis)	Visual	Electronic tag	Electronic tag	Electronic tag
Body location	Fins	Skin	Skin	Skin	Skin	None	Skin/scales	Intra-coelomic	Intra-coelomic	Dorsal skin
Detection and observation range	Close observation (<±20 cm)	Close observation (<±20cm)	Relatively close observation (~30-50cm)	Good (<±1m)	Very good (a few meters)	Relative close observation (depending on body size, of camera resolution, and of optic magnification)	Very close observation or by binocular magnifier use	Limited (±10 cm from antenna)	Weak (<1cm from antenna, does not work in water)	Very weak (<5mm from antenna)
Life-span of method/retention	Short (several days)	Good to very good (generally stable over time for late juveniles and adults)	Excellent (if not rejected, but high risk of rejection)	Limited (several weeks)	Excellent (potentially unlimited)	Duration of the video sequence (several exceptions)	Good (several months if no lost scales) but not immediate	Excellent (if not rejected, potentially unlimited)	Excellent (if not rejected, potentially unlimited)	Excellent (if no rejection, potentially unlimited)
Rereading	Bad (rapid regeneration)	Good	Good (if no partial or complete rejection)	Relatively good (for several weeks, but attenuation over time)	Excellent (stable over the time)	Relatively bad to impossible (between two independent video sequences)	Not easy, and few frequent	Excellent (<1 sec)	Excellent (<1 sec)	Excellent (<1 sec)
# of IDs	Very Poor (2)	Poor to very good (10-30)	Relatively limited to good (6-24)	Limited (6-24)	Currently poor (4)	Limited to good (2-±100 depending on technics, several % of risks of identification errors)	Good to very good (some hundreds)	Excellent (~10^{9-11})	Excellent (~10^{9-11})	Excellent (±10^{9})
Equipment and furnitures	Dissection kit	camera (optional) and a ID software based on picture analysis (optional)	Colourant and injection tools (possibly UV light)	Colourant and injection tools	Mutant needs genetic engineering	Video cameras, computer, programs	Stereo-microscope and dissection kit	Tags, antennas, readers and optionally computer programs	Tags, antennas, readers and optionally computer programs	Tags, antennas, readers and optionally computer programs
Cost by ID tag **Global cost**	0 +	0 + to ++	+ ++	+ ++	++ +++	0 +++ to ++++	0 ++	++ +++	++ +++	++ +++
Developmental stage	Large juvenile and adult	Very early juvenile to adult	Juvenile to adult	Juvenile to adult	Depend on period of activation of fluorescent genes	None (possibility to track early larvae)	Large juvenile and adult	Currently evaluated only with adults	From juvenile >350mg	From juvenile >20mm
Invasive character and social impact	Caudal fin section	None	Use of needle at the level of the skin (generally derm), risk for social behaviour	Use of needle at the level of the skin (generally derm), risk for social behaviour	Risk for social behaviours, risk of physiological impacts	Non invasive	Removing of scales	Use of needle until coelomic cavity, good recovery, no social impact	Use of needle until coelomic cavity, very good recovery, no social impact	Use of needle at the level of the skin

Abbreviations: LF: Low Frequency, HF: High Frequency, VIE: Visible Implant Elastomer, PIT: Passive Integrated Transponder.

Table 2 summarizes the specifications of ID methods for each of the four main contexts in which individual identification is important for zebrafish use in the laboratory. In order to choose the ID method best adapted to a specific context, several criteria must be considered (see summary in Table 3). The first step is to determine the duration of use, typically 'hours or days' versus 'weeks or months'. Some methods have a short lifespan because tags are rapidly rejected or attenuated over time to allow identification. Long-duration methods can be used for short-term research only if the tagging procedure does not affect behaviours, as some more invasive methods may require some time for the fish to recover. The second step is to determine when to identify the fish during the experiment. If not needed during the experimental trial, identification is easier after the observations because fish can then be handled. When a fish is tested alone, there is no need to identify it during the experiment, as its identity can be deduced before or after the test. Live identification is required when a group of individuals is recorded at the same time in the same tank, and there is a need to identify individuals at specific moments and locations during the experiment. Finally, the price, the invasiveness, the impact on individual and social behaviours, and the number of ID codes, are all important criteria in carefully selecting the best method.

Future developments

The development of new technologies could make it possible to accurately track continuously over time many small-sized individuals without manipulation. The new challenges would be to combine different technologies to take advantage of their respective inputs. For instance, combining multi-tracking with RFID technology, through specific algorithms and electronic designs, will allow not only the identification of individuals but also the collection of continuous data, such as behavioural patterns at an individual level. The miniaturization of electronic tags, the improvement of detection distance (particularly using underwater antennae for automatic RFID detection) and the increased capacities and efficiencies of computers are promising developments to fine-tune the observations. Moreover, it could allow the study of zebrafish populations throughout their ontogeny as well as through varied environmental conditions and treatments. Altogether, these new techniques could therefore offer unprecedented tools for acquiring automatically a large amount of data to benefit of large variety of research fields.

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