# *Danio rerio*: the Janus of the bone from embryo to scale

**Massimo Mariotti**[1,2]
**Marta Carnovali**[3]
**Giuseppe Banfi**[1,4]

[1] IRCCS Galeazzi Orthopedic Institute, Milan, Italy
[2] Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy
[3] Gruppo Ospedaliero San Donato Foundation, Milan, Italy
[4] Vita-Salute San Raffaele University, Milan, Italy

Address for correspondence:
Massimo Mariotti
Dipartimento di Scienze Biomediche,
Chirurgiche e Odontoiatriche (sez. Galeazzi)
Università di Milano
Via R.Galeazzi 4
20161 Milano, Italy
E-mail: massimo.mariotti@unimi.it

**Summary**

***Danio rerio* (zebrafish), like the Roman god Janus, is an old animal model which is recently emerged and looks to the future with an increasing scientific success. Unlike other traditional animal models, zebrafish represents a versatile way to approach the study of the skeleton. Transparency of the larval stage, genetic manipulability and unique anatomical structures (scales) makes zebrafish a powerful and versatile instrument to investigate the bone tissue in terms of structure and function. Like Janus, zebrafish offers two different faces, or better, two models in one animal: larval and adult stage. The embryo can be used to isolate new genes involved in osteogenesis by large-scale mutagenesis screenings. The behavior of bone cells and genes in osteogenesis can be investigate by using transgenic lines, vital dyes, mutants and traditional molecular biology techniques. The adult zebrafish represents an important resource to study the pathways related to the bone metabolism and turnover. In particular, the properties of the caudal fin allow to study mechanisms of bone regeneration and reparation whereas the elasmoid scale represents an unique tool to investigate the bone metabolism under physiological or pathological conditions.**

*KEY WORDS: zebrafish; bone; scale; transgenic; mutant; fin; regeneration.*

## Introduction

Janus (Latin: Ianus) is one of the oldest and most important deities of the Roman religion, Latin and Italic. It is usually depicted with two faces, because the god can look to the future and the past, but also because, being the god of the door, can look both inside and outside. *Danio rerio* (zebrafish), like Janus, is an old animal model which is recently emerged and looks to the future with an increasing scientific success.
Bone is the specialized tissue that forms the skeleton of the body in the vertebrates. It is composed mainly of calcium phosphate and calcium carbonate and serves as a storage area for calcium and ions, protect internal organs, support muscles attachment for the movements and participates to the energy metabolism (1).
All these functions are common for all vertebrates, from human to fish. The similarity of the skeletal structure and function between these two far vertebrates has hired zebrafish as animal model to study osteogenesis and bone metabolism processes (2).
Unlike other traditional animal models, zebrafish represents a versatile way to approach the study of the skeleton. Some unique characteristics of this animal model such as transparency of the larval stage, genetic manipulability and unique anatomical structures (scales) makes zebrafish a powerful and versatile instrument to investigate different aspects of the bone tissue in terms of structure and function. Like Janus, zebrafish offers two different faces, or better, two models in one animal: larval and adult stage. In turn, each model can offer different solutions and applications for basic science studies, translational medicine, physiopathology studies and pharmacological studies.

## Zebrafish embryo

The optical transparency of zebrafish embryos allows researchers to keep monitored the internal organs in a way that is not possible in other vertebrates. In addition, small size (1mm of length), wide genetic characterization, external and fast development and low maintenance cost makes zebrafish embryo one of the most powerful and emerging animal tools (3).

## Skeletal tissue in embryogenesis

In zebrafish, as in all bony fishes, two different types of ossification take place: from a cartilaginous scaffold (endochondral ossification) or directly from mesenchymal stem cell precursors (intramembranous ossification). Both phenomena resemble those of the mammalian skeleton (4) (Figure 1).
The development and formation of larval cartilage as well as the adult skeletal anatomy and ossification processes of craniofacial and the axial skeleton have been described in detail (5-7).
In zebrafish, neurocranium (perichondral) and pharyngeal arches (endochondral) develop by replacement of cartilage precursors. While most neurocranial bones ossify relatively late, many endochondral arch bones begin the ossification

Figure 1 - Alizarin red and alcian blue staining of a zebrafish embryo (6dpf).

from 6 to 7 days post-fertilization (dpf). On the other hand, in the development of intramembranous bone, mesenchymal stem cells from neural crests aggregate and differentiate into osteoblasts starting to lay down a mineralized bone matrix. The first dermal bones develop early and include the opercle (3 dpf), parasphenoid (4 dpf), and branchiostegal rays (4 to 5 dpf) whereas dermal plates that cover the skull appear later.
Vertebral column is formed by intramembranous ossification and the vertebral bodies mineralize progressively in a cranio-caudal direction. In zebrafish, vertebral bodies are formed in the absence of cartilage through the mineralization of the notochord (8).
At the cellular level, osteoblasts and osteoclasts of teleosts show high similarity with their counterparts in mammals (9). The transcription factors RUNX2 and Osterix (OSX) are expressed sequentially in the regulation of mammalian osteoblast differentiation and stimulate the transcription of bone matrix proteins (10). During zebrafish osteoblastogenesis the zebrafish homologues of mouse Runx2 (runx2a and runx2b) are typically expressed before osx which drive the expression of bone matrix proteins such as col1a2 and osteonectin (11). These data suggest that zebrafish osteoblast differentiation and function are conserved between fish and humans.
Osteoclastic markers such as matrix metalloproteinase-9 (mmp-9), receptor activator of necrosis factor κ-β (rank), cathepsin-k (Ctsk) and tartrate-resistant acid phosphatase (TRAcP) were found expressed in zebrafish embryos before 5 days post fertilization suggesting that a resorbing activity has a role in the early embryonic development (12).

**Bone live staining**

The first step to study skeletal development is visualizing bone and cartilage. The transparency of the zebrafish larva lends itself to the use of vital dyes since they are non-invasive staining methods. Live animal staining with fluorescent dye have been developed included calcein, alizarin red and quercetin. Vital bone dyes can be administrate to the fish water and rapidly penetrates into zebrafish embryos binding to calcified skeletal structures. Other dyes like alcian blue are used to visualize cartilage matrix but only on fixed samples.
Calcein is a green fluorescent chromophore that specifically binds to calcium. Because the skeletal system is composed of calcified matrix, calcein has been used to mark bone structures and follow their growth during embryo development (13). The absence of toxical effects and a strong fluorescent signal makes calcein the best dye used in live zebrafish bone staining.
Alizarin red stains bone calcified matrix and often is used to distinguish cartilage and bone by counterstaining with Alcian blue (14) (Figure 1). Differently from calcein, which is visible only as green fluorescence, Alizarin red can be visualized as red staining in bright light as well as red emission in fluorescence light. Alizarin red stains early cranial bones at 3 to 4 days of development and continues for many weeks until the adult pattern.
Quercetin is a phytoestrogen present in vegetables and fruits (onion, apple and grape) used to visualize the bone matrix in living teleost. Nevertheless, the live treatment with this molecule has been proved having effects on embryo development (15) and bone physiology (16), therefore it is preferable to use other dyes.
Alcian blue and green are dyes for the proteoglycan components of the extracellular matrix surrounding the chondrocytes. It is used to visualize cartilage patterns both in larvae and in adults. Alcian Blue, after fixation, first stains the chondrocytes from 54 hours of development, but the full staining pattern of early cranial cartilages is evident after 72 hpf (7).

**Bone-specific transgenic lines**

Given the almost complete transparency of the embryos and the relatively short generation time (2-3 months), the researchers have elaborated methods to visualize specific cells during development. The genetic manipulability makes zebrafish well suited for expressing fluorescent proteins (GFP,mCherry,DsRed,Kaede,YFP) under the control of minimal signaling pathway responsive elements (17) using transgenic techniques. Such transgenic fish are particularly useful when fluorescent proteins are expressed in various skeletal cells or tissues. In the last years, many labs have generated different transgenic reporter lines to mark skeletal lineages (cartilage or bone) at different stages of differentiation (18).
About cartilage, collagen type2 is one of the most abundant constituent of this tissue. The expression of *col2a1* in zebrafish has been observed in the developing cartilage, notochord, skin, floor plate, brain, and heart. On the basis of the expression data, transgenic fish have been developed using Col2a1 promoter such as *Tg*(*Col2a1aBAC:mCherry*)*hu5900* (19) and *Tg*(*1.7col2a1a:mCherry-caax*) (20). The cartilage-specific expression of the reporters permits to evidentiate tissue malformation in presence of chemical treatment or diagnose specific diseases like osteoarthritis (19).
About bone tissue, several transgenic lines specific for osteoblasts have been reported in literature. The transcription factor Osterix (Osx) is required for osteoblast differentiation during embryonic development in teleosts (11). Reporter lines

using *osterix*/*sp7* promoter such as Tg(*sp7:EGFP*)*b1212* (21), *medaka* osx-mCherry (22) and Tg(*Ola.Sp7:NLS-GFP*)*zf132* (23) have been made to study the timing of the osteoblast differentiation and the regulation pathways of the embryonic osteogenesis as well as craniofacial bone mapping (24).

Osteocalcin, or bone Gla protein, is a small protein secreted by osteoblasts and found in bone extracellular matrix as hydroxyapatite-binding protein or in the blood as circulating form implicated in the regulation of glucose homeostasis. An *osteocalcin*/*bglap* reporter line, Tg(*Ola.osteocalcin:EGFP*) *hu4008* has been used to demonstrate that bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin (25).

Col10a1 expression is detected in both chondrocytes and osteoblasts in zebrafish, in particular cleithrum, operculum, and parasphenoid, bones generated by intramembranous ossification (26). A stable transgenic zebrafish has been developed using col10a1 promoter that drives fluorescent reporter expression in bone structures. In this case, transgenic fish Tg(*Col10a1BAC:mCitrine*) provides a powerful tool for studying molecular pathways that regulate osteoblast-specific programs not related to a transient chondrocyte development (27).

Osteoclast cells have been described in adult fish as mononucleated and multinucleated and involved in bone remodeling and in developing zebrafish as mononucleated cells only after 20 days post fertilization (28).

Several reporters for osteoclasts in medaka and zebrafish have been developed to produce *in vivo* imaging of timing, function and regulation of the resorbing activity during the embryo development. Osteoclast-specific promoters like tartrate-resistant acid phosphatase (TRAP) and cathepsinK were choosen to generate the transgenic fish.

For example, *TRAP-GFP* transgenic line was used to observe the emergence of osteoclasts and their role in the proper development of different organs (29).

There are also available reporters for osteoclasts using cathepsinK promoter such as *CTSK-DsRed* (29) or *Ctsk YFP* (12) used to examine maturation and migration of osteoclasts in the remodeling processes. To underline the versatility of the transgenesis techniques in fish bone research, *Tg* (ctsk*:mEGFP)* transgenic line has been used in a triple transgenic approach to study the interaction of osteoclasts and osteoblasts upon heat-shock-induced RankL expression. RankL-induced osteoclast differentiation caused a degradation of the mineralized matrix in vertebral bodies and arches miming in medaka an osteoporotic phenotype (30).

The use of transgenic lines have also been applied for joint fate studies. The transgenic line, *trps1*$^{j1271aGt}$ express GFP in joint regions of wild-type fish and it has been used to elucidate the regulatory pathways of the differentiation and organization of joint cells (31).

## Mutants and screenings

Zebrafish was born as model organism for developmental biology in the late ’60 but only in the ’90 was introduced as powerful tool for forward genetic. Indeed, two large-scale “big genetic screens” searching for mutants were carried out in Boston and Tübingen in the early ’90. Due to particular characteristics like small size, rapid development and transparency, the zebrafish embryo is particularly suitable for screening gene mutations that affect the development of heart, kidney, the central nervous system and the motor system (32). The identification of mutants is one of the most important strategies for the study of tissue and organ development. From the mutation, by a forward genetic approach, we can understand the role of the target gene in normal development. More than 50 mutants with defective cartilage and skeletal development were identified from the big screens. In this type of research, a particular advantage in the use of zebrafish comes from the possibility to monitor the skeletal development in real time using fluorescent transgenic embryos. Mutant screens in zebrafish have identified many genes required for early cartilage development, in particular, 109 mutations that perturb pharyngeal arch development. Among them, 59 most severely affect the posterior arches, including the hyoid and five branchial segments that support the gills (33). Other mutations were associated with craniofacial malformations (34).

About bone tissue, four classes of zebrafish bone mutants can be identified: (I) generally less or no bone, (II) ectopic overossification, (III) more perichondral bone, but normal dermal bone (IV) less perichondral bone, but normal dermal bone (35).

Recently, another large-scale ENU-based screen have been performed to find mutants that affect specifically zebrafish bone development (36). Isolated from this screen, *bone calcification slow* (bcs) mutant showed delayed axial vertebra calcification during development without affecting bone formation and maturation. The mutant indicated that different genes regulate different processes like mineralization timing and bone composition.

A mutant fish showing a defect in osteoclastogenesis has been identified. The zebrafish mutant *panther* possess a mutation in the zebrafish orthologue of the mammalian c-fms gene encoding the M-CSF receptor (37, 38). c-fms knockdown mice were affected by osteoclast deficiency and osteopetrosis (39). In contrast, zebrafish *panther* mutant does not exhibit skeletal anomalies despite the c-fms gene is expressed in osteoclasts. Nevertheless, a defective number of osteoclasts was observed during fin regeneration in *panther* (38).

The zebrafish mutant *stocksteif*, defective in cyp26b1 gene, shows severe over-ossification of the vertebral column. In zebrafish and mouse, cyp26b1 was found expressed in osteoblasts of the pre-vertebrae sites and regulates the bioavailability of retinoic acid, a crucial molecule for axial osteogenesis (40).

The zebrafish mutant nob (no bone) shows complete lack of skeletal mineralization caused by a mutation in the ectonucleotidase (*entpd5)* gene, where the encoded protein has a crucial role in modulating the levels of nucleoside tripsophates and diphosphates (41).

Another example of osteogenesis-related zebrafish mutant is *dragonfish (dgf)*. The mutation affects ectonucleoside pyrophosphatase/phosphodiesterase 1 (Enpp1), a protein that is crucial for supplying extracellular pyrophosphate. Zebrafish *enpp1* mutants are characterized by ectopic calcifications in several soft tissues (skin, cartilage, heart, intracranial space and notochord). Enpp1 gene regulates pathways involved in phosphate homeostasis and mineralization, regardless of the osteoblast number or differentiation (42).

The use of the mutants is a powerful tool to study the role of the genes involved in the development of the vertebrate skeleton.

Several mutants isolated from big screens have shown high correspondence with human patients affected by bone dis-

eases in terms of pathological manifestations. Considering the extreme similarity between human and fish about morphology and function of the mineralized tissues, it is not surprising that mutations in orthologue genes give rise to a similar pathological and clinical manifestation in humans and fish.

For example, generalized arterial calcification of infancy (GACI) and some cases of pseudoxanthoma elasticum (PXE) have recently been linked to ENPP1. Human patients with ENPP1 mutation have shown ectopic mineralization in several soft tissues and arteries, increasing the risk of cardiovascular diseases. Zebrafish ENPP1 mutants is characterized by ectopic calcifications in several soft tissues indicating that the role of this gene is conserved (42).

Raine Syndrome (RNS) is an autosomal recessive genetic disease with generalized osteosclerosis, facial dysmorphism and intracerebral calcifications. Increased bone density and cranial hypermineralization suggested that the mutation is associated to a gene involved in the mineralization process. In fact, it has been found the target gene (FAM20C) which encodes a secreted kinase that phosphorylates substrates involved in the mineralization like osteopontin (43). Zebrafish homozygous mutant in the gene fam20b shows similar morphological alterations found in human RNS patients (44).

Mutations in CYP26B1 cytochrome P450, family 26, subfamily B, polypeptide 1 are associated with radiohumeral fusions and other skeletal and craniofacial anomalies. This gene encodes a member of the cytochrome P450 superfamily which functions as a critical regulator of the level of all-trans retinoic acid. Some clinical cases of craniosynostosis have shown important phenotypic similarities with zebrafish mutant *stocksteif*, which possesses an inactivating mutation in the same gene (45).

Congenital vertebral malformations (CVM) occur in 1 in 1000 live births and in many cases can cause spinal deformities, such as scoliosis, and result in disability and distress of affected individuals. Many severe forms of the disease, such as spondylocostal dystostosis, are recessive monogenic traits affecting somitogenesis, however the etiologies of the majority of CVM cases remain undetermined. Here we demonstrate that morphological defects of the notochord in zebrafish can generate congenital-type spine defects. We characterize three recessive zebrafish *leviathan*/*col8a1a* mutant alleles (*m531, vu41, vu105*) that disrupt *collagen type VIII alpha1a* (*col8a1a*), and cause folding of the embryonic notochord and consequently adult vertebral column malformations (46).

## Adult zebrafish

Zebrafish larva offers several unique solutions to approach the study of the skeletal system. Small size, transparency and rapid development makes easy the analysis of the osteogenic processes. However, bone turnover, reparation and remodeling of the adult bone tissue cannot be found during embryo development because the fish, at this stage, does not possess mature bone. In addition, regenerating fins and scales represent unique characteristics of this animal model. Therefore, the adult zebrafish represents an important resource to study the pathways related to the bone metabolism and turnover. Adult bone shows similar properties to human counterpart, suggesting that it may be used with success as a model to study mineralization characteristics of the human Haversian system, as well as human bone diseases. In fact, several human adult bone diseases like secondary osteoporosis can be reproduced in adult zebrafish in order to generate simply pathological model systems to dissect the pathophysiological mechanisms and design new therapy (47).

## Skeletal defects and malformations

The exposure to various chemical agents during sexual development shows defects in the cranial and axial skeleton in the adult stage. For example, sublethal administration of dioxin in early embryogenesis or during sexual determination assessed the effects later in adulthood where the most frequent manifestation was a scoliosis caused by malformation of individual vertebrae (48). With this approach, the analysis of the adult stage results crucial for the knowledge of the pathogenetic mechanisms in several conditions.

## Advanced imaging techniques

Several advanced imaging resources have been used in small animal models like rodents. In the last years, tomographic techniques like Time-Gated Optical Projection Tomography (TGOPT), were used in adult zebrafish to reconstruct three dimensionally the internal structure without chemical contrast (49). Regarding the analysis of the skeletal system, whole zebrafish can be scanned by µCT, permitting the determination of BMD of total body as well as specific bone (i.e., a section of the spinal column) from a single scan. Whole-body µCT scans also permitted a 3D reconstruction of the zebrafish skeletal system in order to detect defects or differences in bone structures after particular dietary supplementation like strontium (50). X-ray radiography have been already introduced in zebrafish studies to detect abnormalities in skeletal anatomy and bone morphology (51).

## Bone-related mutants

Despite the use of the mutants is inevitably linked to the larval stage, the analysis of the adult stage of a specific mutant may be essential in the characterization of the phenotype. That is the case of the zebrafish mutant *Chihuahua* which was isolated in a forward-genetics screen of adult fish using X-ray searching for skeletal abnormality (51). Heterozygous fish shows abnormal bone growth, altered vertebral shape, defective mineralization and frequent fractures. These characteristics can be found in human patients affected by heterozygous *osteogenesis imperfecta* (OI), a rare genetic disease caused by a mutation in the gene *col1a1*. The *chihuahua* is generated by an heterozygous missense mutation G390D in *col1a1a*, an homologue of human *col1a1*. The Glycine residue changed in the mutation match in the conserved Gly-X-Y motif, the same residue mutated in the human patients. Thus, the adult zebrafish mutant *chihuahua* can be introduced as model of dominant human *osteogenesis imperfecta* useful to study new therapeutic approaches such as stem cells transplantation.

The generation of a fish mutant named *casper* has introduced a transparent adult zebrafish where the transplantation of stem/progenitor cells or tumor cells can be easily followed by *in vivo* imaging techniques (52). In this mutant the skeleton

can be visualize without the use of dyes. Casper has been created by crossing two mutants with defective pigmentation. The *nacre* mutant has a complete lack of melanocytes whereas the spontaneous mutant *roy orbison* (*roy*) has a complete lack of iridophores, uniformly pigmented eyes, sparse melanocytes, and a translucency of the skin. The double mutant for *nacre* and *roy* was named *casper* for its ghost like phenotype. The complete absence of melanocytes and iridophores in both embryo and adult stage makes the body of the fish almost entirely transparent.

**Fin fracture and regeneration**

Adult teleosts, including zebrafish, possess the ability to regenerate different organs and tissues following amputation or injury, including caudal fin (53). The caudal fin is composed by radial bone structures named rays (lepidotrichia), soft tissues and vessels. The ray is acellular and mineralize following an intramembranous ossification (54) whereas, at the base of the fin, the endoskeleton is formed by endochondral ossification (55). The resection of the fin at the level of the endoskeleton does not involve a regeneration, whereas amputation of the dermal fin rays results in complete regeneration of the original structures in almost 20 days (53).
Zebrafish caudal fin, also because of its accessibility and transparency, has been introduced to investigate both reparative and regenerative capacity of the adult bone tissue.
After fin amputation, mature osteoblasts dedifferentiate and form part of the blastema. Resident osteoblasts inhibit the expression of late bone differentiation genes and induce the expression of genes involved in proliferation and survival of progenitor cells (56).
Fin regeneration in teleosts is not ideal model to study the repair system of human bone fractures because it involves important and multiple tissue removal, rather than local bone injury.
A caudal fin fracture model has been developed to understand how zebrafish skeletal cells respond in a situation comparable to a mammalian long-bone fracture. In this case is not required a regeneration program but rather a repair process much more similar to a wound healing (57). This model will help to elucidate the mechanisms involved in adult bone repair.
The dedifferentiation of adult osteoblasts is not restricted to fin regeneration but also occurs during fin fractures repair and skull injuries. In all cases, mature osteoblasts surrounding the injury site downregulate the expression of differentiation genes whereas upregulate early genes used in the pre-osteoblast state and reactivate the cell proliferation (58).
The transparency of the adult caudal fin encourages the use of technical resources like vital staining, transgenic lines and antibodies.

**Scales**

The adult zebrafish is covered with dermal bone structures named “elasmoid scales” which mechanically protect the body. The scale is a bone lamella structured in two main mineralized layers composed by fibrillary collagen and hydroxyapatite crystals in different proportion (59). The episquamal (external) side has high mineralized component respect to the fibrillary one, whereas the hyposquamal side is the opposite, with a plywood architecture much more similar to mammalian lamellar bone (60, 61).
Anatomically, the scale is characterized by concentric ridges (circuli) and grooves (radii) which radiate from the central focus to the edges of the scale (59).
The scale matrix is synthesized by the scleroblasts which resemble to osteoblasts in terms of embryonal origin and function [104]. Scleroblasts express several osteoblastic markers and are organized in a monolayer at the surface of both side of the scale. In particular, the episquamal osteoblast covering the external side are devoted to the deposition of the mineralized layer. The hyposquamal osteoblasts, located in the internal side, are responsible of the deposition of the plywood-like collagen architecture which constitutes the basal plate. Alkaline phosphatase (ALP) is expressed in scleroblast (62) with a strong upregulation during the early phase of cell differentiation at the mineralization sites (63).
In teleosts as well in mammals, the tartarate-resistant acid phosphatase (TRAcP) and Cathepsin K activity was associated to an osteoclast-mediated resorbing activity (9). Indeed, mononuclear and multinuclear osteoclasts were found on the episquamal side of scales in association with resorption sites (62). Multinucleated osteoclasts resorbing the scale matrix have also been identified by means of electron microscopy in fish in physiological (64) and pathological conditions (47).
Scales can be removed with a forceps without causing suffering in fish and a new scale is regenerated in approximately 14 days. Explanted scale can be used as read out system for bone metabolism evaluating biochemical and cellular markers, bone matrix architecture and deposition (65). To do that, several assays can be performed such as biochemical assays for enzymatic activity of ALP and TRAP, histochemical staining with calcein and/or alizarin red (Figure 2), immunohistochemical staining with antibodies (ex:. Zn5), gene expression evaluation by real-time PCR. A live double staining with alizarin red and calcein (Figure 3) has been recently developed to measure the mineralization rate of the scale after treatment with chemical compounds or drugs (47). The transparency and the size of the scale facilitates manipulation, treatment and observation of the results under a stereomicroscope.
Since an adult animal possess about 200 scales, the statistical power of a scale analysis is undoubtedly high.

Figure 2 - Calcein staining of a zebrafish scale.

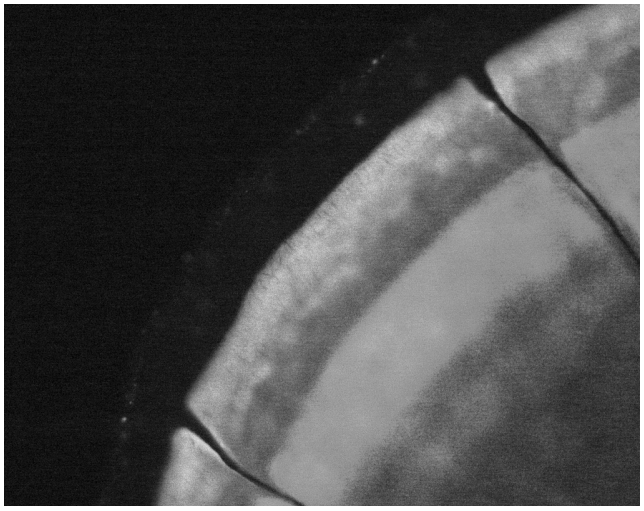

Figure 3 - Sequential alizarin red/calcein double staining of a zebrafish scale.

A complete scale, with its associated cells, can be cultured *in vitro* for several days and represents an *ex-vivo* model in which the behaviour of fish bone cells (scleroblasts and osteoclasts) can be tested *in vitro* on its original surface (66). This makes them ideal sample to study mineralization and remodeling mechanisms.

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