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# Multiscale imaging of metastasis in zebrafish

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## **Abstract**

Cancer progression to metastatic dissemination is responsible for ~90% of death in patients. Tremendous efforts have been made to understand primary tumor growth, cancer genetics and clonal evolution, but also secondary sites of colonization. Intravital imaging technologies are instrumental in understanding key steps of the metastasis cascade, which are believed to be therapeutically relevant targets. However, these remain cumbersome in mouse models. Recent work has demonstrated zebrafish's unique ability as an experimental metastasis model for the dynamic study of cancer progression at the single cell level. Its compatibility with state-of-the art imaging techniques and biophysical approaches allows to probe the interaction of tumor cells with their microenvironment and to monitor fast and rare cellular events at high spatio-temporal resolution. In this review, we highlight the multiple benefits of zebrafish as an alternative metastasis preclinical model from an imaging standpoint.

## **Zebrafish: a complementary model to mouse for tracking tumor cells at high-resolution**

Cancer progression is a dynamic multistep process which includes (1) tumor cells breaking out of their organ of origin and invading the surrounding stroma, (2) entry into the circulatory system termed intravasation, (3) blood borne transport to distant organs and extravasation and (4) colonization of the parenchyma of the targeted organs and formation of clinically detectable lesions termed metastases [1]. Metastatic cells are capable of surviving multiple obstacles along these steps to form secondary tumors at distance, through the process of metastatic colonization [2]. These steps are highly dynamic and high-resolution **intravital imaging (see glossary)** is as an essential tool to shed light on some unknown mechanisms [3]. Metastatic progression results from the coordinated action of heterogeneous cell populations where single cell imaging offers an unprecedented mechanistic understanding. . An animal model that allows to probe, *in vivo*, single-cell behaviors with functional assays is thus likely to reveal important mechanisms caused by tumor heterogeneity [4]. It is essential to understand the key steps and identify molecular components involved at each step may of the metastatic cascade as this may provide important therapeutic opportunities [5].

67 Mouse models have been the gold standard in cancer research since the early 1980s  
68 because they have close to ~99% gene homology to humans, a similar overall body  
69 organization, and conserved organ function and immune system . Although they  
70 provide important insights into cancer progression, they have, however, several  
71 intrinsic limitations. Maintenance of mouse facilities is costly and high statistical  
72 sampling with datasets including a large number of individuals remains cumbersome.  
73 Intravital imaging of metastasis in mouse models is very instructive, yet it remains  
74 challenging and time-consuming. Such imaging requires surgical implantation of  
75 optical windows as well as adapted expensive microscopy to adapt to breathing  
76 motion of the anesthetized animal [6–8]. Fine understanding of the multiple steps of  
77 the metastasis cascade would strongly benefit from the use of state-of-the-art  
78 imaging approaches that can be combined with adapted and relevant animal models  
79 where metastasis, and its **microenvironment**, can be modeled realistically.

80 Among such alternative animal models, ***Danio rerio*** (zebrafish) possess qualities  
81 that are very complementary to traditional murine models [9]. Zebrafish breeding  
82 produces a high number of embryos with a fully external development. Embryos are  
83 naturally transparent during the embryonic and larval stages, which makes them  
84 compatible with any kind of imaging-based research strategy and provides increased  
85 throughput and resolution. Zebrafish have ~70% gene homology with humans, and  
86 most organs and cellular functions are conserved. **Transgenesis** (the process by  
87 which genetic material is transferred by engineering techniques from one organism to  
88 another) allows to rapidly generate lines where fluorescent proteins controlled by  
89 specific promoters allow to document the behavior of specific cell types (Table 1-2).  
90 Transgenesis also allows to modify the microenvironment, create cancer models and  
91 cell fate reporters that are relevant to cancer research. In particular, there is now a  
92 vast number of cell-type specific promoters that can be used to drive Cas9 or cDNAs  
93 in virtually any microenvironmental cell type which is exceptionally powerful in  
94 comparison to the mouse model [10]. In addition, the structural organization of its  
95 body is highly stereotyped all along its development and, when combined with high-  
96 throughput imaging, enables spatial projection of single cells over several embryos  
97 [11,12] or adults [13]. Most importantly, in terms of histology and genomics, zebrafish  
98 tumors and metastatic foci histologically and genomically resemble the human  
99 pathology and are driven by a growing list of oncogenes and tumor suppressors.  
100 Zebrafish can be treated with carcinogens and mutagens that produce tumors that

resemble human cancers. Altogether, this makes the zebrafish a highly relevant model for cancer research with imaging and statistical power.

In this Opinion, we first review the main advantages (and limitations) of the zebrafish model and its application as a preclinical cancer model. We further highlight key findings in cancer research that have been obtained when combining zebrafish cancer models with highly resolutive imaging and biophysical approaches to emphasize the emerging role of the zebrafish as a model for imaging, and thereby understanding, metastatic progression (Text Box 1).

### **Zebrafish: an optimal model for modeling and imaging tumor metastasis.**

Over the past two decades, the zebrafish has emerged as an alternative model for cancer biology. It has been mainly used to study **oncogenomics** and the interaction of tumor cells with the microenvironment, in particular the **juxtacrine** and **paracrine** signaling; or used as an *in vivo* drug screening platform [9]. Further, its compatibility with state-of-the-art high-resolution imaging has been elemental in deciphering major developmental programs, including early embryogenesis, somitogenesis, neurogenesis and angiogenesis [14]. This argues in favor of using zebrafish as a model for easy intravital imaging of cancer progression. Here, we list several advantages of this model for tracking tumor metastasis with imaging approaches.

#### *Optical clarity*

The zebrafish embryo is transparent during the first week of its development, which makes it perfectly suited for intravital imaging. This property has been elemental in its emergence as a reference model in developmental biology (Figure 1). However, as it develops, melanogenesis will induce the development of melanophores, iridophores and xanthophores which are incompatible with imaging since they are autofluorescent. Finally, transparency will be lost as the adult stripe pattern appears after a month [15]. The combination of a mutation in the *miffta* locus (*nacre*) with the spontaneous mutant *roy orbison* which lacks iridophores has generated the *casper* line which is transparent with black non-autofluorescent eyes throughout his lifetime [16]. The *casper* background has now become a standard in the imaging field within the zebrafish community and makes it a first-choice for imaging-based strategies (Box) to study several pathologies, including tumor progression (Figure 1).

#### *Engineering of spontaneous cancer models with tunable microenvironment*

Several spontaneous tumor models have now been described and associated with the use of imaging strategies to analyze cancer progression at the single cell level in fully **syngeneic models**. They were generated by the tissue-specific expression of oncogenes and/or tumor suppressor and include skin carcinoma [17], melanoma [17,18], hepatocellular carcinoma [19], rhabdomyosarcoma [20] and several models of brain cancers [21–23] (Table 1). In these examples, intravital imaging (Box) was used to probe dynamically cell behaviors, tumors growth or gene expression and signaling pathways activation during tumor progression. Particularly, the easiness of transgenesis associated with the large number of tissue-specific promoter driving tissue-restricted expression makes the zebrafish model ideal for the on-demand engineering of preclinical cancer models. Relevant fluorescent markers can be specifically expressed either in endogenous tumor cells or within cells which is essential for intravital imaging of the dynamic interaction of cancer cells with their microenvironment [24]. Furthermore, the gene editing technology CRISPR is now routinely used in the zebrafish model and applied for cell-specific gene knock-in or knock-out [25]. This is a field and particularity that will continue growing in the future and which makes the zebrafish model uniquely powerful in comparison to other models where this remains challenging, slow and costly.

#### *Tumor cell grafting and immuno-deprived models*

Interestingly, the zebrafish embryo has no functional adaptive immune system for up to 10 days, which makes it ideal for short-term **xenograft** experiments. Although one limitation is that multiple stromal cell types are lacking at this stage, tumor cells can be either injected within the stroma between **intersomitic vessel** [26], the yolk sac [27], or the hindbrain [28]. Although this aspect still remains challenging, the immune system can be chemically suppressed for favoring the growth of tumor cells from other species (15~30 days) [26]. More recently, fully immuno-compromised zebrafish lines have been generated [29,30]. They are compatible with xenografts either from human and mouse cancer cell lines or even from patient derived tumors (PDX) for periods up to 60 days. One limitation of the zebrafish model is that they are classically maintained at 28°C which is incompatible with the growth of mammalian cells, including human cells. However, such limitation is now resolved with the creation of immuno-compromised lines that can be maintained at 37°C upon a slow temperature increase for smooth acclimation [30]. This major advance demonstrates the possibility to follow tumor growth *in vivo* using intravital imaging in a more

physiologically relevant context. Furthermore, with **photoconversion** (the process by which light can chemically change the structure of fluorescent proteins and thereby change their emission spectra), it was possible to perform single cell lineaging within tumors to analyze the dynamic behavior of individual cells within tumors [30]. The combination of immuno-compromised lines compatible with human tumors engraftment maintained at 37°C and the imaging compatible *casper* genetic background, makes the zebrafish a unique model from an imaging point of view. Yet, there are some remaining limitations. Xenografting of mammalian cells requires an immune-compromised background and thus fails at recapitulating the complex interaction of tumor cells with the immune system. To circumvent this drawback, a zebrafish melanoma cell line, ZMEL-1, has been isolated from a spontaneous melanoma model and thus allows to interrogate metastatic progression of melanoma in a syngeneic situation. Such development allows the transplant of genetically manipulated (and fluorescently-labeled) melanoma cells for long-term cancer progression model in a full immuno-competent organism. Further development of these strategies associated with long-term imaging should allow the study of whole process from primary tumor initiation until secondary organ colonization [13] (Figure 1A). Alternatively, in order to bypass primary tumor growth and study arrest of circulating tumor cells (CTCs) and subsequent metastatic steps, any type cells, either from fish or mammals tumor cell lines or from primary tumors can be directly injected in the vascular system [31] (Figure 1B).

## **Intravital Imaging of the early steps of metastatic dissemination in zebrafish**

### *Tumor initiation and growth*

Using genetic engineering in zebrafish, the tissue-specific expression of oncogenes such as Ras<sup>G12V</sup> or BRAF<sup>V600E</sup> was driven to study tumor initiation within the organs of interest [18,21,22,32,33]. More specifically, intravital live imaging demonstrated the role of inflammation and tumor/immune cells interaction in cancer initiation and showed, for example, that neutrophils respond to wounds (biopsy) by amplifying cell proliferation of nearby pre-neoplastic cells [32,34]. In a very elegant study, a model of spontaneous melanoma expressing the neural crest marker *crestin:egfp*, which is reactivated in melanoma cells, allowed to track melanoma progression using intravital imaging at the single cell level from the first detectable melanoma cell to a

fully developed tumor. Doing so, an epigenetic signature of melanoma progression emerged which is also conserved in human melanoma [33].

#### *Basement membrane breakage*

A second important step of the metastatic cascade is stroma invasion by tumor cells after basement membrane breakage (Figure 2) [1]. Such step has recently been documented in a study of spontaneous skin and melanoma cancers (Table 1) in zebrafish embryos expressing specifically collagen I:eGFP. By combining intravital imaging with **correlative light and electron microscopy** (CLEM, Box), authors have demonstrated the presence of wounds within the **extracellular matrix** (ECM) which could be used to favor tumor cell escape (Figure 2) [17]. This demonstrates that combining transgenic lines of tissue-specific fluorescent ECM components with fluorescently labelled tumor cells makes zebrafish an excellent model to image and study early steps of tumor dissemination at high resolution (Figure 2).

#### *Stromal invasion and interaction with the immune microenvironment*

Using xenografted fluorescent tumor cells in the stroma between intersomitic vessels of larva expressing an endothelium-specific eGFP (*fli1a:eGFP*, Table 2), it was demonstrated that cells with an amoeboid-like behavior invaded sparsely the stroma and migrated independently of vascular paracrine signals. Mesenchymal-like tumor cells migrated more as clusters and along vessels. However, while mesenchymal tumor cells could intravasate between endothelial junctions, amoeboid-like tumors cells required tumor induced vascular remodeling to intravasate at sites of vascular openings (Figure 2) [26]. Inflammatory signals triggered by the immune system is known to favour tumor progression [35]. Visualizing the dynamics of tumor cell interaction with immune cells from the microenvironment, either directly or through paracrine signals, is elemental in understanding of the role of the immune system during tumor progression. Several lines expressing fluorescent proteins in specific lineages of immune cells are available and have been already used to understand the interactions between tumor and immune cells (Table 2). Of note, several lines generated for the specific lineage tracing of B, T lymphocytes as well as natural killer cells are available but have not been used in the context of tumor progression so far [36–40]. Intravital imaging associated with cell tracking and CLEM, which combines the advantages of *in vivo* live imaging with ultrastructural analysis, has documented that neutrophils and macrophages infiltrate tumors through wounds in the ECM, thus promoting tumor associated inflammation and tumor growth in skin carcinomas or



melanomas [17,34]. Similarly, intravital imaging showed that neutrophils infiltrate the brain during tumor initiation and promote tumor growth in a CXCR1 & 2-dependent manner [41]. Xenograft of tumor-associated macrophages (TAM) from mouse and humans, including patients, with mouse or human tumor cell lines in *Tg(fli1a:eGFP)* zebrafish embryos suggested that TAM, and more particularly M2 macrophages, promote tumor cell intravasation [42]. Recent work that combined intravital imaging with a **Cre/LoxP** strategy shows that melanoma cells directly interact with TAM, which induces cytoplasmic transfer from TAM to tumor cells and promote their dissemination [28]. Such exchange is likely to occur through **tunneling nanotubes** [43]. It was also showed that both endogenous fluorescent macrophages and neutrophils are present within xenografted primary tumors and their **micrometastasis**. Morpholino-mediated *in vivo* RNA interference demonstrated that only neutrophils were required to establish metastatic colonies [44].

#### *Tumor angiogenesis*

The endothelial driven expression of fluorescent protein achieved with a *fli1a* promotor has been a major tool in studying zebrafish angiogenesis [45] from single cell dynamics to cell signaling in processes such as sprouting, branching and remodeling [46]. Not surprisingly, early studies aiming to track tumor progression in zebrafish embryos exploited this experimental advantage and established protocols to track tumor angiogenesis upon xenografting [47]. In two studies, high-resolution confocal imaging showed vasculature remodeling toward xenografted tumor cells, most likely due to tumor VEGF secretion [26,44]. This process could be inhibited by VEGFR inhibitors, including Sunitinib (Sutent©) which is used as a neo-angiogenesis inhibitors in patient [44]. In another study, confocal intravital live imaging of blood vessels reorganization was coupled to **microangiography** and demonstrated the role of blood flow in the stimulation of VEGF-induced neoangiogenesis [48].

#### *Priming of metastatic niches*

Extracellular vesicles (EVs), including exosomes, mediate long distance cell-cell communication and are shed from tumor cells to prime pre-metastatic niches, which will form a favorable “soil” for disseminating tumor cells [49]. While several studies exploit the advantages of the mouse model to document such impact, tracking EVs *in vivo* in mice remains cumbersome [50]. Using high-speed imaging combined with particle tracking and CLEM, we recently used the zebrafish embryo to probe EV behaviour *in vivo*. Doing so, we demonstrated that tumor EVs disseminate actively

through the bloodstream, exploit its hemodynamics (the forces generated by blood flow) and are taken up by endothelial cells and patrolling monocytes. This will then promote the formation of the pre-metastatic niches and metastasis that can now be probed accurately in zebrafish embryos [27]. Further usage of CLEM is likely to provide unprecedented insights into the biology of tumor EVs. Altogether, this demonstrates that the zebrafish is an optimal model for imaging and tracking the early steps of the metastasis cascade.

### **Intravital Imaging of the late, intravascular, steps of metastatic dissemination in zebrafish**

Once intravasation is completed, circulating tumor cells (CTCs) are disseminated to distant organs. The endothelium-specific expression of fluorescent markers in the embryo associated with the injection of fluorescently-labelled CTCs and fast intravital imaging at the single cell level allows the study of their arrest, adhesion and extravasation [11,51,52] (Table 2, Figure 2). During blood borne transport, CTCs are subjected to **hemodynamics forces** that dictate CTC arrest sites [53,54]. Using **optical tweezers**, we have set up a method to accurately measure hemodynamic forces in the living embryo [55]. This approach has defined a threshold flow value in the zebrafish embryo below which CTC arrest is promoted. Interestingly, such discovery made through the imaging and biophysical characterization of CTC has then been validated in mouse models and human patients [11]. This permissive flow is also found in the capillary beds of organs and matches the main arrest sites observed for disseminating cancers [2,54]. This further emphasizes the benefits of the zebrafish as a preclinical cancer model. CTC arrest is thus controlled by the limited adhesive energy of CTCs and the forces from blood flow [12], further emphasizing that biomechanical cues, that can be probed in zebrafish (Box) are important factors regulating the metastatic cascade. More recently, **lattice light sheet microscopy** enabled to document with live imaging in 3D human breast cancer cells from cell lines rolling along blood vessels [56], previously observed using confocal imaging [51]. However, this is likely not a general feature of cancer cells, as it was not observed for other cell lines [11,12]. With fast intravital imaging, we were able to discriminate between low and high energy adhesion receptors involved in early CTC arrest and **shear stress** (the component of stress which is orthogonal to a material surface caused by the fluid flow)-resistant stable adhesions respectively

[12]. In addition, it is now also possible to inject CTC directly in the vasculature of adult zebrafish. This approach was used to probe the role of mature vasculature architecture in CTC arrest [57]. It appeared the architecture did not impact CTC arrest, reinforcing the idea that occlusion is not necessary for CTC arrest [11,12,57]. CTC clusters have now emerged as more metastatic than single CTCs [58,59]. However, this is unexpected as larger clusters should have a higher propensity to get stuck in larger vessels and thus should reach less efficiently organ capillary beds. The transit of CTC clusters was studied in the zebrafish embryo and it was demonstrated that CTC clusters can actively rearrange making them highly deformable [60]. These examples demonstrate how intravital imaging in zebrafish allows to probe the dynamic behavior of CTCs in a physiologically relevant context. Altogether, this demonstrates that multiple intravascular steps of the metastatic dissemination can be studied using single-cell imaging of circulating tumor cells and their microenvironmental partners.

### **Intravital imaging of metastatic extravasation, dormancy and outgrowth in zebrafish**

In order to colonize new organs, CTCs should exit the circulatory system [1]. Both embryos and adults have a highly stereotyped vasculature that allows the efficient screening of a high number of individuals and to easily pool data in order to generate statistically relevant datasets using **heatmapping** (a graphical representation of data sets where the intensity of a given measure is color-coded) [13,31]. Intravital imaging in zebrafish allowed to assess different extravasation efficiencies of different cell lines [51]. Using RNAi-mediated knockdown, it is possible to quantify the involvement of specific adhesion receptors in extravasation. This approach highlighted the specific role of the integrin ITGB1 in extravasation [11,12,51], while other receptors such as CD44 are not required [12]. With intravital time lapse imaging, we and other observed an alternative route for cancer extravasation owing to endothelial remodeling either in large non-occluding vessels [11] or in smaller capillary-sized ISVs [51]. Using CLEM, we observed that upon single or clustered CTC arrest, flow induced an active endothelial remodeling to restore flow, thus passively extravasating CTCs [11]. This mechanism is slower (6~10h) than diapedesis, which might explain why it has not been observed in mouse model with challenging long-time imaging. The ability of the endothelium to remodel in the zebrafish embryo was previously described with a

similar imaging strategy during perivascular niche establishment upon hematopoietic stem cell (HSC) homing, a structure which is also conserved in mouse fetal liver, the major HSC reservoir [61]. Endothelial remodeling was observed in mouse brain capillaries after CTC arrest at sites of extravasation with higher flow velocities [11]. Imaging at metastatic sites showed that sprouting vessels promote metastatic outgrowth by creating a perivascular niche which induces **dormancy** (state of cancer cell characterized by cell cycle exit and quiescence) exit [62]. Adhesion receptors are also required to promote dormancy exit and proliferation disseminated cancer cells adhesion in colonized organs [62]. The zebrafish model allows to look at each individual step and thus to discriminate between extravasation *per se* and the subsequent foci growth. After extravasation, a subset of cells with higher potential to form metastatic colonies, named tumor initiating cells (TIC), will give rise to micrometastasis which will eventually turn into clinically detectable macrometastasis [1]. Zebrafish embryos also emerges as a potential model to assess the metastasis forming potential of TIC within a given extravasated cancer cell population. A recent study demonstrated as a proof of principle that the TIC population of cells could induce more metastasis after extravasation than their non-TIC counterparts [63]. Zebrafish could thus be used to screen rapidly and extensively for the TIC potential among CTC populations but also to specifically target genes and screen for their TIC inducing potential. The *casper* model associated with syngeneic ZMEL-1 melanoma cells has also been used to study metastatic colonies formation with intravital imaging from single extravasation to macrometastasis [57]. This approach could be further used using immunocompromised *casper* models to assess the long term metastatic potential of PDX [29,30]. In conclusion, the zebrafish can thus be used as an optimal imaging platform for further dissecting extravasation and metastatic outgrowth mechanism.

### **Concluding remarks**

The zebrafish model is now emerging as an alternative to mouse preclinical models for cancer research. Screening strategies have long been used in zebrafish in particular to study its development. This past decades has proved that it could also be used as a preclinical screening platform for anti-tumoral drugs where *zebrafish 'avatars' are likely to guide treatment plans for cancer patients* [9]. Recent preclinical examples include the use perphenazine to treat T cell acute lymphoblastic leukaemia

and the combination of olaparib and temozolomide for rhabdomyosarcoma [64,30]. These strategies have led to the identification of new compounds such as all-trans retinoic acid and leflunomid for adenoid cystic carcinoma and melanoma treatment respectively, which have reached phase I trial [65,66]. The zebrafish model has also emerged as an alternative to immune-deficient mouse models for zebrafish patient-derived xenografts (zPDX) [29]. It was recently used as a platform to screen for chemotherapy efficiency. This suggests that this model could also be further used to fine tune cancer treatment depending on primary or CTC specificities in an aim of personalized medicine. When combined with resolutive imaging technologies, such strategy is likely to unravel unexpected anti-metastatic compounds and mechanisms. However, zebrafish, as most animal models, is still unable to fully recapitulate cancer progression. Zebrafish cancer models are now limited to inducing primary tumors through xenografts or mimicking cancer dissemination using experimental metastasis methods. Nevertheless, the advances in genome editing have now enabled the generation of spontaneous cancer models in zebrafish which should be used to further study metastatic dissemination (Table 1). With the improvement in microscopy time and spatial resolution and automated image analysis, easy CRISPR-mediated knock-out strategies either in cell lines or primary cells or in living model organism, it is tempting to anticipate that the zebrafish model could further evolve into a platform for the whole genome screening of candidate genes involved in each step of tumor progression and the metastatic cascade. The emergence of *in vivo* compatible super resolutive imaging technologies *in vivo*, such as **3D STED** or lattice light sheet with adapted optics with improved spatial and time resolution respectively, will undoubtedly put further forward the zebrafish as a first-choice imaging model in preclinical cancer research. Recent development in Brillouin microscopy [67], that appears to be compatible, and thus powerful, with small organisms such as the zebrafish [68], now allow to probe mechanical properties of tissues during tumor and metastatic progression in zebrafish. We anticipate that the use of these new intravital imaging techniques and biophysical approaches will help to document in detail at the single cell or molecule level the highly dynamic events of cancer cells/microenvironment interactions involved in cancer progression but also the clonal genomic and epigenetic evolution of disseminated cancer cells.

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## References

- 1 Lambert, A.W. *et al.* (2017) Emerging Biological Principles of Metastasis. *Cell* 168, 670–691
- 2 Massagué, J. and Obenauf, A.C. (2016) Metastatic colonization by circulating tumour cells. *Nature* 529, 298–306
- 3 Follain, G. *et al.* (2017) Seeing is believing – multi-scale spatio-temporal imaging towards in vivo cell biology. *J Cell Sci* 130, 23–38
- 4 Blackburn, J.S. and Langenau, D.M. (2014) Zebrafish as a model to assess cancer heterogeneity, progression and relapse. *Dis. Model. Mech.* 7, 755–762
- 5 Steeg, P.S. (2016) Targeting metastasis. *Nat. Rev. Cancer* 16, 201–218
- 6 Entenberg, D. *et al.* (2018) A permanent window for the murine lung enables high-resolution imaging of cancer metastasis. *Nat. Methods* 15, 73–80
- 7 Dondossola, E. *et al.* (2018) Intravital microscopy of osteolytic progression and therapy response of cancer lesions in the bone. *Sci. Transl. Med.* 10, eaao5726
- 8 Warren, S.C. *et al.* (2018) Removing physiological motion from intravital and clinical functional imaging data. *eLife* 7, e35800
- 9 Cagan, R.L. *et al.* (2019) Modeling Cancer with Flies and Fish. *Dev. Cell* 49, 317–324
- 10 Kim, I.S. *et al.* (2017) Microenvironment-derived factors driving metastatic plasticity in melanoma. *Nat. Commun.* 8, 14343
- 11 Follain, G. *et al.* (2018) Hemodynamic Forces Tune the Arrest, Adhesion, and Extravasation of Circulating Tumor Cells. *Dev. Cell* 45, 33–52.e12
- 12 Osmani, N. *et al.* (2019) Metastatic Tumor Cells Exploit Their Adhesion Repertoire to Counteract Shear Forces during Intravascular Arrest. *Cell Rep.* 28, 2491–2500.e5
- 13 Heilmann, S. *et al.* (2015) A Quantitative System for Studying Metastasis Using Transparent Zebrafish. *Cancer Res.* 75, 4272–4282
- 14 Kimmel, C.B. *et al.* (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203, 253–310
- 15 Rawls, J.F. *et al.* (2001) How the Zebrafish Gets Its Stripes. *Dev. Biol.* 240, 301–314
- 16 White, R.M. *et al.* (2008) Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. *Cell Stem Cell* 2, 183–189
- 17 van den Berg, M.C.W. *et al.* (2019) Proteolytic and Opportunistic Breaching of the Basement Membrane Zone by Immune Cells during Tumor Initiation. *Cell Rep.* 27, 2837–2846.e4
- 18 Patton, E.E. *et al.* (2005) BRAF Mutations Are Sufficient to Promote Nevi Formation and Cooperate with p53 in the Genesis of Melanoma. *Curr. Biol.* 15, 249–254

- 451 19 Evason, K.J. *et al.* (2015) Identification of Chemical Inhibitors of  $\beta$ -Catenin-Driven  
452 Liver Tumorigenesis in Zebrafish. *PLOS Genet.* 11, e1005305
- 453 20 Langenau, D.M. *et al.* (2007) Effects of RAS on the genesis of embryonal  
454 rhabdomyosarcoma. *Genes Dev.* 21, 1382–1395
- 455 21 Ju, B. *et al.* (2015) Oncogenic KRAS promotes malignant brain tumors in  
456 zebrafish. *Mol. Cancer* 14, 18
- 457 22 Mayrhofer, M. *et al.* (2017) A novel brain tumour model in zebrafish reveals the  
458 role of YAP activation in MAPK- and PI3K-induced malignant growth. *Dis. Model.*  
459 *Mech.* 10, 15–28
- 460 23 Modzelewska, K. *et al.* (2016) MEK Inhibitors Reverse Growth of Embryonal  
461 Brain Tumors Derived from Oligoneural Precursor Cells. *Cell Rep.* 17, 1255–1264
- 462 24 Suster, M.L. *et al.* (2009) Transgenesis in Zebrafish with the Tol2 Transposon  
463 System. In *Transgenesis Techniques: Principles and Protocols* (Cartwright, E. J.,  
464 ed), pp. 41–63, Humana Press
- 465 25 Li, M. *et al.* (2016) Zebrafish Genome Engineering Using the CRISPR–Cas9  
466 System. *Trends Genet.* 32, 815–827
- 467 26 Stoletov, K. *et al.* (2007) High-resolution imaging of the dynamic tumor cell–  
468 vascular interface in transparent zebrafish. *Proc. Natl. Acad. Sci.* 104, 17406–  
469 17411
- 470 27 Hyenne, V. *et al.* (2019) Studying the Fate of Tumor Extracellular Vesicles at  
471 High Spatiotemporal Resolution Using the Zebrafish Embryo. *Dev. Cell* 48, 554–  
472 572.e7
- 473 28 Roh-Johnson, M. *et al.* (2017) Macrophage-Dependent Cytoplasmic Transfer  
474 during Melanoma Invasion In Vivo. *Dev. Cell* 43, 549–562.e6
- 475 29 Fior, R. *et al.* (2017) Single-cell functional and chemosensitive profiling of  
476 combinatorial colorectal therapy in zebrafish xenografts. *Proc. Natl. Acad. Sci.*  
477 114, E8234–E8243
- 478 30 Yan, C. *et al.* (2019) Visualizing Engrafted Human Cancer and Therapy  
479 Responses in Immunodeficient Zebrafish. *Cell* 177, 1903–1914.e14
- 480 31 Follain, G. *et al.* (2018) Using the Zebrafish Embryo to Dissect the Early Steps of  
481 the Metastasis Cascade. In *Cell Migration* pp. 195–211, Humana Press, New  
482 York, NY
- 483 32 Feng, Y. *et al.* (2012) Live Imaging of Tumor Initiation in Zebrafish Larvae  
484 Reveals a Trophic Role for Leukocyte-Derived PGE2. *Curr. Biol.* 22, 1253–1259
- 485 33 Kaufman, C.K. *et al.* (2016) A zebrafish melanoma model reveals emergence of  
486 neural crest identity during melanoma initiation. *Science* 351, aad2197
- 487 34 Antonio, N. *et al.* (2015) The wound inflammatory response exacerbates growth  
488 of pre-neoplastic cells and progression to cancer. *EMBO J.* 34, 2219–2236
- 489 35 Wellenstein, M.D. and de Visser, K.E. (2018) Cancer-Cell-Intrinsic Mechanisms  
490 Shaping the Tumor Immune Landscape. *Immunity* 48, 399–416



491 36 Langenau, D.M. *et al.* (2004) In vivo tracking of T cell development, ablation, and  
492 engraftment in transgenic zebrafish. *Proc. Natl. Acad. Sci.* 101, 7369–7374

493 37 Kasheta, M. *et al.* (2017) Identification and characterization of T reg–like cells in  
494 zebrafish. *J. Exp. Med.* 214, 3519–3530

495 38 Page, D.M. *et al.* (2013) An evolutionarily conserved program of B-cell  
496 development and activation in zebrafish. *Blood* 122, e1–e11

497 39 Liu, X. *et al.* (2017) Zebrafish B Cell Development without a Pre–B Cell Stage,  
498 Revealed by CD79 Fluorescence Reporter Transgenes. *J. Immunol.* 199, 1706–  
499 1715

500 40 Moore, F.E. *et al.* (2016) Single-cell transcriptional analysis of normal, aberrant,  
501 and malignant hematopoiesis in zebrafish. *J. Exp. Med.* 213, 979–992

502 41 Powell, D. *et al.* (2018) Cxcr1 mediates recruitment of neutrophils and supports  
503 proliferation of tumor-initiating astrocytes in vivo. *Sci. Rep.* 8, 13285

504 42 Wang, J. *et al.* (2015) Novel Mechanism of Macrophage-Mediated Metastasis  
505 Revealed in a Zebrafish Model of Tumor Development. *Cancer Res.* 75, 306–315

506 43 Hanna, S.J. *et al.* (2019) Tunneling nanotubes, a novel mode of tumor cell-  
507 macrophage communication in tumor cell invasion. *J. Cell Sci.* 132,

508 44 He, S. *et al.* (2012) Neutrophil-mediated experimental metastasis is enhanced by  
509 VEGFR inhibition in a zebrafish xenograft model. *J. Pathol.* 227, 431–445

510 45 Isogai, S. *et al.* (2003) Angiogenic network formation in the developing vertebrate  
511 trunk. *Dev. Camb. Engl.* 130, 5281–5290

512 46 Betz, C. *et al.* (2016) Cell behaviors and dynamics during angiogenesis.  
513 *Development* 143, 2249–2260

514 47 Nicoli, S. and Presta, M. (2007) The zebrafish/tumor xenograft angiogenesis  
515 assay. *Nat. Protoc.* 2, 2918–2923

516 48 Nicoli, S. *et al.* (2010) MicroRNA-mediated integration of haemodynamics and  
517 Vegf signalling during angiogenesis. *Nature* 464, 1196–1200

518 49 Peinado, H. *et al.* (2017) Pre-metastatic niches: organ-specific homes for  
519 metastases. *Nat. Rev. Cancer* 17, 302–317

520 50 Hyenne, V. *et al.* (2017) Going live with tumor exosomes and microvesicles. *Cell*  
521 *Adhes. Migr.* 11, 173–186

522 51 Stoletov, K. *et al.* (2010) Visualizing extravasation dynamics of metastatic tumor  
523 cells. *J Cell Sci* 123, 2332–2341

524 52 Asokan, N. *et al.* (2017) Continuous high-resolution in vivo imaging reveals  
525 tumor-specific dissemination in an embryonic zebrafish xenograft model. *bioRxiv*  
526 DOI: 10.1101/215921

527 53 Wirtz, D. *et al.* (2011) The physics of cancer: the role of physical interactions and  
528 mechanical forces in metastasis. *Nat Rev Cancer* 11, 512–522

529 54 Chambers, A.F. *et al.* (2002) Metastasis: Dissemination and growth of cancer  
530 cells in metastatic sites. *Nat. Rev. Cancer* 2, 563–572

531 55 Harlepp, S. *et al.* (2017) Hemodynamic forces can be accurately measured in  
532 vivo with optical tweezers. *Mol. Biol. Cell* 28, 3252–3260

533 56 Liu, T.-L. *et al.* (2018) Observing the cell in its native state: Imaging subcellular  
534 dynamics in multicellular organisms. *Science* 360, eaaq1392

535 57 Benjamin, D.C. and Hynes, R.O. (2017) Intravital imaging of metastasis in adult  
536 Zebrafish. *BMC Cancer* 17, 660

537 58 Aceto, N. *et al.* (2014) Circulating Tumor Cell Clusters Are Oligoclonal Precursors  
538 of Breast Cancer Metastasis. *Cell* 158, 1110–1122

539 59 Cheung, K.J. *et al.* (2016) Polyclonal breast cancer metastases arise from  
540 collective dissemination of keratin 14-expressing tumor cell clusters. *Proc. Natl.*  
541 *Acad. Sci.* 113, E854–E863

542 60 Au, S.H. *et al.* (2016) Clusters of circulating tumor cells traverse capillary-sized  
543 vessels. *Proc. Natl. Acad. Sci.* 113, 4947–4952

544 61 Tamplin, O.J. *et al.* (2015) Hematopoietic Stem Cell Arrival Triggers Dynamic  
545 Remodeling of the Perivascular Niche. *Cell* 160, 241–252

546 62 Ghajar, C.M. *et al.* (2013) The perivascular niche regulates breast tumour  
547 dormancy. *Nat. Cell Biol.* 15, 807–817

548 63 Chen, J. *et al.* (2016) Efficient extravasation of tumor-repopulating cells depends  
549 on cell deformability. *Sci. Rep.* 6, 19304

550 64 Gutierrez, A. *et al.* (2014) Phenothiazines induce PP2A-mediated apoptosis in T  
551 cell acute lymphoblastic leukemia. *J. Clin. Invest.* 124, 644–655

552 65 White, R.M. *et al.* (2011) DHODH modulates transcriptional elongation in the  
553 neural crest and melanoma. *Nature* 471, 518–522

554 66 Mandelbaum, J. *et al.* (2018) Zebrafish blastomere screen identifies retinoic acid  
555 suppression of MYB in adenoid cystic carcinoma. *J. Exp. Med.* 215, 2673–2685

556 67 Bevilacqua, C. *et al.* (2019) Imaging mechanical properties of sub-micron ECM in  
557 live zebrafish using Brillouin microscopy. *Biomed. Opt. Express* 10, 1420–1431

558 68 Schlüßler, R. *et al.* (2018) Mechanical Mapping of Spinal Cord Growth and Repair  
559 in Living Zebrafish Larvae by Brillouin Imaging. *Biophys. J.* 115, 911–923

560 69 Kanada, M. *et al.* (2014) Endothelial cell-initiated extravasation of cancer cells  
561 visualized in zebrafish. *PeerJ* 2, e688

562 70 Asokan, N. *et al.* (2017) Continuous high-resolution in vivo imaging reveals  
563 tumor-specific dissemination in an embryonic zebrafish xenograft model. *bioRxiv*  
564 DOI: 10.1101/215921

565 71 Verweij, F.J. *et al.* (2019) Live Tracking of Inter-organ Communication by  
566 Endogenous Exosomes In Vivo. *Dev. Cell* 48, 573–589.e4

567 72 Goetz, J.G. *et al.* (2014) Endothelial cilia mediate low flow sensing during  
568 zebrafish vascular development. *Cell Rep.* 6, 799–808

569 73 Goetz, J.G. *et al.* (2015) Using correlative light and electron microscopy to study  
570 zebrafish vascular morphogenesis. *Methods Mol. Biol. Clifton NJ* 1189, 31–46

- 74 van den Berg, M.C.W. *et al.* (2019) Proteolytic and Opportunistic Breaching of the Basement Membrane Zone by Immune Cells during Tumor Initiation. *Cell Rep.* 27, 2837-2846.e4
- 75 Ariotti, N. *et al.* (2015) Modular Detection of GFP-Labeled Proteins for Rapid Screening by Electron Microscopy in Cells and Organisms. *Dev. Cell* 35, 513–525
- 76 Lagendijk, A.K. *et al.* (2017) Live imaging molecular changes in junctional tension upon VE-cadherin in zebrafish. *Nat. Commun.* 8, 1402
- 77 Grashoff, C. *et al.* (2010) Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* 466, 263–266
- 78 Anton, H. *et al.* (2013) Pulse propagation by a capacitive mechanism drives embryonic blood flow. *Dev. Camb. Engl.* 140, 4426–4434
- 79 Johansen, P.L. *et al.* (2016) Optical micromanipulation of nanoparticles and cells inside living zebrafish. *Nat. Commun.* 7, 10974
- 80 Perton, F. *et al.* (2019) Wrapped stellate silica nanocomposites as biocompatible luminescent nanoplateforms assessed in vivo. *J. Colloid Interface Sci.* 542, 469–482

## Glossary

**Intravital Imaging:** refers to any type of microscopy that is performed on living organisms (embryos and adults)

**Microenvironment:** defines neighboring tissues composed of stromal cell types and associated extracellular matrix and fluids

**Oncogenomics:** refers to studies aiming to characterize cancer-associated genes by focusing on genomic, epigenomic and transcript modifications during tumor progression.

**Juxtacrine:** refers to short distance cell communication though direct interaction between a receptor on a cell and a counter-receptor on the counterpart cell.

**Paracrine:** refers to short distance cell communication though biochemical messengers.

**Syngeneic models:** they refer to animal models where tumor tissues are derived from the same genetic background as a given animal strain. The animals have an intact immune system.

**Xenograft:** refers to a graft (cells, tissues or organs) whose origin is from a different species than the recipient one.

**Intersomitic vessel:** These vessels sprout and develop from the aorta (dorsal) of the

**Extracellular matrix:** refers to 3D network of macromolecules which provides a biological, chemical and physical support to cells.

**Cre-LoxP :** refers to an engineered recombination mechanism that uses a site-specific recombinase technology for deleting, inserting, translocating or inverting DNA sequences within cells.

**Tunneling nanotubes:** are protrusions that extend from the plasma membrane of cells and favour contact with and transfer of material to other cells over 100  $\mu\text{m}$ .

**Microangiography:** is a technique in which blood vessels are visualized by the use of contrasting agents (that can be fluorescent) coupled to microscopy

**Optical tweezers:** is a microscopy-based biophysical method which uses a highly focus beam to trap objects within the focal plan.

**Lattice light sheet microscopy:** is a microscopy modality which uses a sheet of structured light sheet to illuminate successive single planes. This is coupled to highly sensitive EM-CCD camera for single plane acquisition. This methods achieves fast 3D imaging.

**3D STED:** stands for 3D stimulated-emission-depletion. This is confocal microscopy modality based on the deactivation of fluorophores which are out of the focus on the main activating laser by a second doughnut shaped laser in order to achieve higher spatial resolution.

**FLIM-FRET:** stands for Fluorescence Lifetime Imaging Microscopy - Fluorescence Resonance Energy Transfer. This is a microscopy modality is based on the energy transfer between close fluorophores and is used to study protein-protein interaction

**Table 1: Major zebrafish lines with spontaneous cancer available**

| <b>Zebrafish line</b>                             | <b>Cancer model</b>                 | <b>Ref</b> |
|---|-------------------------------------|------------|
| krt4:kalTA4-ERT2; UAS:HRAS <sup>G12V</sup>        | Squamous cell carcinoma (inducible) | [17]       |
| krt19: kalTA4-ERT2; UAS:HRAS <sup>G12V</sup>      | Basal skin carcinoma (inducible)    | [17]       |
| kita:GAL4; UAS:HRAS <sup>G12V</sup>               | Melanoma                            | [17]       |
| kita:Ras <sup>G12V</sup> eGFP                     | Melanoma                            | [32]       |
| mitfa:BRAF <sup>V600E</sup> tp53 <sup>-/-</sup>   | Melanoma                            | [18]       |
| fabp10:pt-beta-catenin                            | Hepatocellular carcinoma            | [19]       |
| rag2:KRAS <sup>G12D</sup>                         | Rhabdomyosarcoma                    | [20]       |
| krt5:KRAS <sup>G12V</sup>                         | Brain cancer (VZ)                   | [21]       |
| gfap:KRAS <sup>G12V</sup>                         | Brain cancer (VZ+P)                 | [21]       |
| zic4:GAL4TA4,UAS:GFP-KRAS <sup>G12V</sup>         | Brain cancer (VZ)                   | [22]       |
| sox10:NRAS <sup>wt/Q61L</sup> tp53 <sup>-/-</sup> | CNS-PNET                            | [23]       |

VZ = ventricular zone, P = parenchyma, CNS-PNET = primitive neuroectodermal tumors of the CNS

**Table 2: Zebrafish lines available for intravital imaging of cancer progression**

| Zebrafish line  | Line specificity                                 | Process studied                                     | Ref           |
|---|--|---|---------------|
| Tumor angiogenesis  |  |   |               |
| fli1a:EGFP  | Endothelium-specific expression of EGFP          | Vessel sprouting and remodeling                     | [26,44]       |
| Immune system interaction                                 |  |   |               |
| mpeg1:EGFP  | Macrophage-specific expression of EGFP           | Macrophages EV uptake                               | [27]          |
| mpeg1:mCherry   | Macrophage-specific expression of mCherry        | Macrophages/cancer cell interaction                 | [17]          |
| lyz:DsRed2  | Neutrophil-specific expression of mCherry        | Neutrophils/cancer cell interaction                 | [17]          |
| mpx:EGFP  | Neutrophil-specific expression of EGFP           |   | [44]          |
| Invasion  |  |   |               |
| krt19:col1α2-GFP  | Skin-specific expression of collagen I           | Matrix degradation                                  | [17]          |
| Hematogenous metastasis                                   |  |   |               |
| fli1a:EGFP  | Endothelium-specific expression of EGFP          | CTC circulation, arrest, adhesion and extravasation | [11,12,51,62] |
| kdrl:EGFP   |  |   | [69,70]       |
| kdrl:mCherry  | Endothelium-specific expression of mCherry       |   | [60]          |
| kdrl:Has.HRAS-mCherry                                     |  |   | [70]          |
| Cell tracking and lineage tracing                         |  |   |               |
| roy <sup>a9/a9</sup><br>nacres <sup>w2/w2</sup>           | Optically-clear                                  | Syngeneic graft of ZF melanoma cell line            | [13]          |
| BRAF <sup>V600E</sup> tp53 <sup>-/-</sup><br>crestin:EGFP | Spontaneous melanoma<br>Neural crest cell marker | Spontaneous melanoma single cell tracing            | [33]          |
| roy <sup>a9/a9</sup><br>nacres <sup>w2/w2</sup>           | Optically-clear<br>immunodeficient               | Tumor cell tracing from xenograft                   | [30]          |

|  |  |  |  |
|--|--|--|--|
| prkdc <sup>-/-</sup> , il2rga <sup>-/-</sup> |  |  |  |
|--|--|--|--|

# **Text Box 1: Zebrafish: a toolbox for imaging metastasis *in vivo*.**

Zebrafish became a popular animal model in developmental biology for the multiple benefits it provides in terms of single-cell imaging that is achieved thanks to its optical transparency. Since the embryos can be easily mounted in batches and kept alive for several hours, they can be imaged from the entire animal (using stereomicroscopy) to single cells (confocal or, recently, lattice light sheet imaging). Both embryos and adults have a highly stereotyped vasculature that allows the efficient screening of a high number of individuals and to easily pool data in order to generate statistically relevant datasets using heatmapping [13,31]. Such property, when combined with ***time-lapse stereomicroscopy*** can be used to track the early steps of metastatic dissemination [11,12,31]. While metastatic events are rare and thus difficult to document in a mouse model (which requires the grafting of optical windows and advanced two-photon microscopy set-ups), ***confocal microscopy*** is sufficient to track individual tumor cells [11,51], immune and stromal cells [28] or tumor-derived extracellular vesicles [27]. When combined with ***high-speed scanners and spinning-disk technologies***, such imaging allows the tracking of CTCs and tumor-derived extracellular vesicles at unprecedented spatio-temporal resolution [11,27,71]. The creation of an adult transparent line, Casper [16], allows now to track single metastatic cells in the process of establishing metastatic foci [10,57] or during tumor invasion [26], without using ***two-photon excitation microscopy***. Recent work also demonstrated that high-resolution can be obtained when studying metastasis or tumor invasion in zebrafish embryos. In particular, the relatively simple anatomy of the zebrafish embryo allows to develop ***correlative light and electron microscopy*** strategies [72,73] that allow to dissect delivery of extracellular vesicles [27], basement membrane breakage [74], or metastatic extravasation [11] at nanoscale resolution. Recent development in protein tagging allowed the creation of transgenic lines that are likely to facilitate correlative imaging [75]. ***Real time and selective plane illumination microscopy (SPIM)*** for up to 30 hours, and on several embryos simultaneously, allows to track metastatic dissemination both at high-resolution and high-throughput [70]. Further developments are likely to push the field forward and provide new insights. For example, ***lattice light sheet microscopy*** enabled to

document with live imaging of metastatic arrest and extravasation from blood vessels [56].

In addition, zebrafish imaging compatibility makes it perfectly suited for non-invasive imaging-based biophysical approaches. Recently, **Brillouin microscopy** was used in embryo to study the viscoelastic properties of the ECM [67] and to mechanical variations during spinal cord development and repair [68]. Another interesting tool is the zebrafish line expressing a FRET-based tension sensor in the endothelium [76]. This strain allows to detect endothelium tension changes using **FLIM-FRET imaging**. With accurate calibration, this tension sensor was previously shown to measure forces at the single molecule level in focal adhesions of migrating fibroblasts [77]. Accurate probing of hemodynamic forces can be performed using **optical tweezers** [55,78], that has proven to be relevant in the context of circulating tumor cells [11]. A similar tool has been used to trap and deliver nanoparticles in the zebrafish circulation [79]. Altogether, these studies demonstrate that the zebrafish embryo emerges as a powerful model to study the impact of biophysical cues on tumor progression. In addition, recent work demonstrates that the zebrafish embryo is likely to unlock some barriers in the field of **drug delivery** where one can track biomaterials, that can be functionalized, in living zebrafish embryos [80].

## Figure legends

**Figure 1 – Available zebrafish experimental cancer models.** (A) 1- Tumor cells can be engrafted within the embryo or the larva in a vascularized stroma, either in the yolk sac or between intersomitic vessels (ISV) or in the hindbrain for short term (1~7 dpi) or long term experiments (up to 20~30 dpi). Embryo transparency is maintained either by chemically inhibiting melanogenesis with 1-phenyl-2-thiourea or using transparent lines such as *casper*. 2- Tumor cells can be engrafted in adult *casper* zebrafish either in the flank or in the eye. Mammalian tumors cell lines of patient-derived xenograft (PDX) can be grafted in casper fish with an immunocompromised genetic background or zebrafish syngeneic ZMEL-1 melanoma cells can be used for re-grafting. (B) Cancer cells in suspension (either from cell lines, from primary tumors or patient CTCs) can be injected directly in the circulation in the duct of Cuvier as an experimental metastasis model mimicking tail vein injection in mouse. The hematogenous dissemination and metastatic colonization can be observed using



intravital imaging. Early CTC arrest is observed 5 min post-injection (mpi), stable arrest at 3h post-injection (hpi) and extravasation between 6-24 hpi and metastatic seeding after 24hpi.

**Figure 2 – Imaging cancer progression *in vivo* in zebrafish.** Scheme of cancer progression. (1) Tumor grows in a primary organ. Tumors cells from cell lines (MDA-MB-231 expressing GFP, left) or primary tumors (breast tumor PDX stained with DiI) can be engrafted in the stroma and their growth monitored *in vivo* over time. Adapted from [30]. (2) Tumor cells break out of the primary tumor and invade the stroma. Basement membrane wound are used by immune cells to infiltrate the tumor and promote growth. Electron microscopy of a macrophage (red) traversing the basement membrane (green). Adapted from [17]. (3) Invading cancer cells interact with stromal resident cells. Stromal engrafted A375 melanoma cells interacting directly with endogenous macrophages in Tg(mfap4:tdTomato-CAAX) zebrafish line. Adapted from [28]. (4) Tumors secreted extracellular vesicles (EVs) which are entering blood circulation. Engrafted zebrafish melanoma cell (ZMEL-1) expressing Syntenin2-EGFP in a Tg(*fli1a:Gal4*; *UAS:RFP*) embryo secretes EVs toward blood vessels. Adapted from [27]. (5) After invasion, tumors cells reach vessels and enter blood circulation by intravasation. MDA-MB-435 breast cancer cell expressing DsRed engrafted in a Tg(*fli1a:eGFP*) embryo entering blood stream. Adapted from [26]. (6) Cells and EVs are transported by the blood flow to a flow permissive region found usually in organ capillary beds. ZMEL EVs stained with MemBright Cy5 in flow with red blood cells (red) and arrested and uptaken by the endothelium (green) in a Tg(*fli1a:eGFP*; *gata1:RFP*) line. Adapted from [27]. (7) CTCs arrest intravascularly by physical occlusion or active adhesion to the vascular wall. Mouse mammary cancer cells D2A1-LifeAct-RFP injected in the blood stream of Tg(*fli1a:eGFP*). CTCs arrested in the arterio-venous junction in the zebrafish embryo tail (left). Correlative light and electron microscopy (CLEM, refers to the combination of light/intravital microscopy and electron microscopy to probe the ultrastructure of cellular events previously imaged *in vivo*) shows a direct interaction between CTC (red) and endothelial (green) protrusions (right). Adapted from [11]. (8) CTC extravasate through diapedesis between endothelial cell junctions or by active endothelial remodeling. EM picture shows CTC surrounded by an endothelial sheet (left). 3D segmentation shows that CTC are fully enwrapped in the endothelial sheet (top

right). 3D tomography shows close proximity between the CTC (red) and the endothelium (green, bottom right). Adapted from [11]. (9) Blood borne EVs are taken up by endothelial cells, released in the parenchyma of distant organs and taken up by resident cells to promote the formation of a premetastatic niche. Labelled ZMEL-1 EV (red) injected in the circulation of a Tg(*mpeg1:eGFP*) is taken up by a monocyte expressing EGFP (green). Adapted from [27]. (10) The established premetastatic niche observed close to blood vessels promotes the growth of secondary colonies at metastatic sites. MDA-MB-231-mCherry human breast tumor cells colonies grow next to vessels sprouts in a perivascular niche which promotes dormancy exit in Tg(*fli1a:eGFP*) line. Adapted from [62]. (11) Upper panel: extravasated tumor cells initiate tumor formation by forming micrometastasis which will eventually form clinically detectable macrometastasis. Mouse mammary cancer cells D2A1-LifeAct-RFP injected in the blood stream of Tg(*fli1a:eGFP*) have extravasated and invaded the caudal stroma. Micrometastases are detected 24 hours after injection (top). 3D segmentation shows that colonies are extravascular. Bottom panel: heatmaps show major extravasation sites in the caudal plexus. Adapted from [11]. Scale bars: 1 = 0.25 cm, 2 = 500 nm, 3 = 30  $\mu$ m, 4 = 10  $\mu$ m, 5 = 20  $\mu$ m, 6 = 50  $\mu$ m; 7 = 50  $\mu$ m (left); 1  $\mu$ m (right), 8 = 2  $\mu$ m, 9 = 5  $\mu$ m, 10 = 100  $\mu$ m; 11 = 25  $\mu$ m.

## Highlights

- Zebrafish emerges as a cancer preclinical model
- Spontaneous cancer models can be generated in zebrafish
- Zebrafish allows intravital imaging with high spatial and temporal resolution of cancer cell interaction with the microenvironment
- Zebrafish is compatible with single cell analysis within tumors and metastases
- Genome editing technologies can be used easily in zebrafish to probe the role of the microenvironments in cancer progression.

## Outstanding Questions

- Will *in vivo* genome-wide imaging-based screening strategies identify new key genes in tumor progression?
- Can we correlate tumor cell behaviors observed in zebrafish with pathological features in patients (MRI, scanner, PET-SCAN)?
- Will zebrafish coupled to imaging help better understand cancer clonal genomic and epigenetic evolution, dormancy and dissemination?
- Will high-resolution microscopy (3D STED, lattice light sheet with adapted optics) and FLIM-FRET deepen the understanding of cancer cell/microenvironment interaction at the single cell or molecule level?
- Is zebrafish likely to provide a new tool to test nanomedicine-based drug delivery and assess patient personalized medicine ?

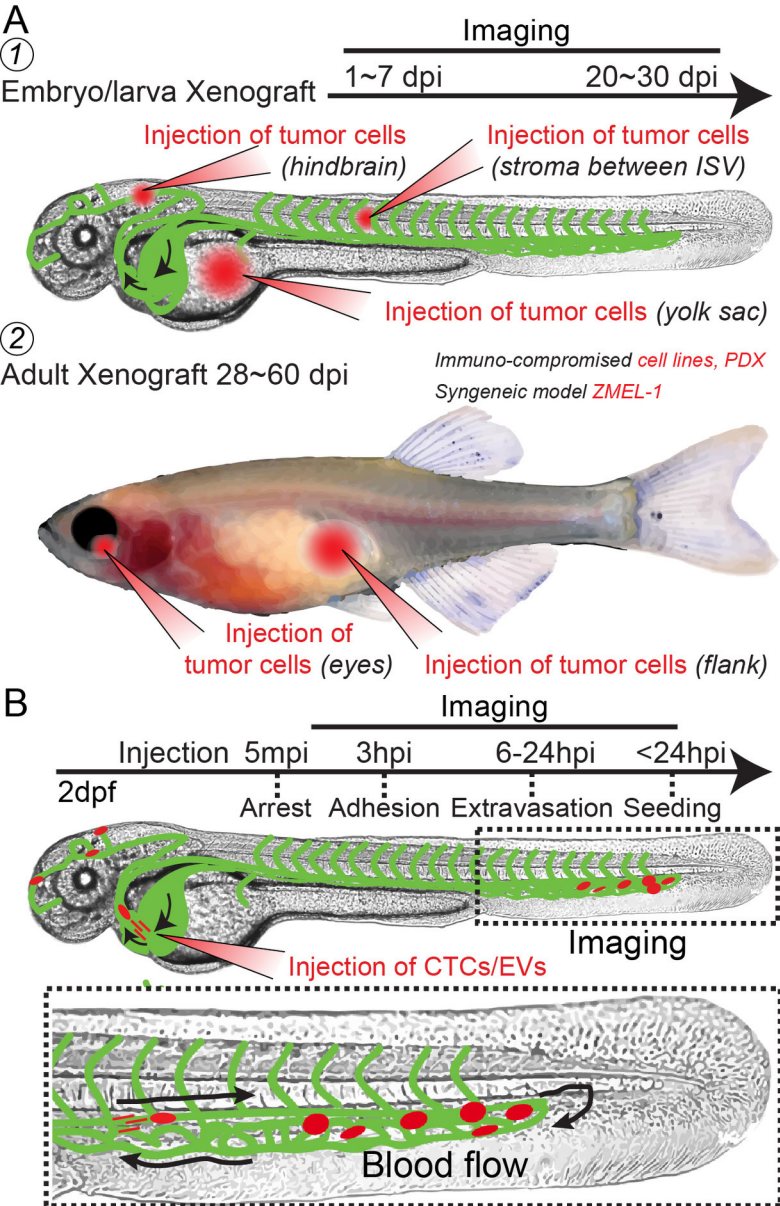


Figure 1\_Osmani et al.



