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Extracellular Vesicles Catching the Light in Zebrafish

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Abstract (120 words max)

Extracellular Vesicles circulate in body fluids of all organisms and participate in intercellular cross-organ communication. Tracking and understanding these nanosized objects have been hampered by limited resolutive imaging and adapted animal models. The use of zebrafish embryos now allows to probe them at unprecedented spatio-temporal resolution, using light and electron microscopy. This enables the study of endogenous physiological EVs and pathological EVs side by side, and further unravel their biogenesis mechanisms, biodistribution, and their target cells throughout the organism. These developments will contribute to a better understanding of the *in vivo* (patho)physiology of EVs

Key-words: zebrafish, exosomes, extracellular vesicles, *in vivo*, high resolution imaging, circulation.

Highlights (3-5, 900 characters max)

Multiple lines of evidence indicate that circulating extracellular vesicles (EVs) are involved in cell-cell communication in physiologic and pathologic contexts.

While their cell biology is now better understood *in vitro*, how they behave at a sub-cellular level in complex organisms remained difficult to assess due to the lack of animal models adapted to their small size.

Zebrafish embryos allow to visualize circulating EVs from their cells of origin to their target cells and to further explore their fate and function during development or tumor progression.

In the future, it will certainly contribute to expand the knowledge on EVs at the whole organism level.

Glossary (450 words max)

Extracellular vesicle: Generic term encompassing all types of lipid bilayered vesicles released outside of cells; **Exosomes:** extracellular vesicles originating from Multi-vesicular bodies; **Microvesicles:** extracellular vesicles emanating from the plasma membrane; **Multi-Vesicular Body:** MVB, late endosome filled with intraluminal vesicles which can either be degraded or secreted as exosomes; **Mosaic expression:** the presence of multiple

genotypes in a single population of cells, in the context of this review specifically referring to the expression of an EV marker of interest in only a fraction of the cells within a given tissue; **Hagen-Poiseuille equation:** Physical law describing the flow forces of a viscous fluid along a cylindrical pipe; **Patrolling macrophages:** Macrophages present in the circulation of zebrafish, similar to mouse and human patrolling monocytes; **Scavenger receptors:** Membrane bound receptors involved in the removal of objects from the blood circulation; **CD63:** Tetraspanin protein enriched very broadly in exosomes and small microvesicles; **pHluorin:** pH sensitive GFP variant, whose fluorescence is quenched in acidic compartments; **Transcytosis:** Transport of molecules or vesicles across an epithelial or endothelial barrier; **YSL:** the Yolk Syncytial Layer is a transient, highly secretory, epithelial monolayer covering the yolk that forms early during zebrafish development and plays an important role in cell fate specification and morphogenesis; **Caudal vein plexus:** well-perfused venous endothelial network at the tail end of the fish (see figure 1), formed via active angiogenic sprouting and intussusception, as well as fusion, starting at 25 hours post fertilization.

INTRODUCTION

Extracellular Vesicles (EVs) are released by most cell types and can elicit a phenotypic response in the secreting cell itself (autocrine), in neighbouring or distant receiving cells. Over the past two decades, EVs have emerged as versatile mediators of cell-to-cell communication, linked to many important physiological and pathological processes, notably development and cancer progression [1]. Initially characterized and studied using biochemical approaches, research on EVs can now benefit from recent advances in high resolution optical microscopy and *in vivo* models, notably the translucent zebrafish embryo [2,3] (Box 1), which allows the analysis of EVs at single-vesicle level *in vivo*. Descriptive observations at this level are likely to open new avenues of research and to generate novel hypotheses in the field [4]. Here, we review the recent developments in EV biology provided by the use of zebrafish as model organism and discuss a number of open questions that they are likely to resolve.

BIOGENESIS AND SECRETION

EVs are a heterogeneous collection of membranous vesicles enriched in subsets of proteins, genetic material and lipids, reflecting the cell type and physiological state of the cell of origin. They find their origin either in late endosomes - in which case they are referred to as '**exosomes**' (50-150nm in size) - or bud directly from the plasma membrane (PM) – typically referred to as '**microvesicles**' (100~1000nm in size) [5]. Besides these two main members of the EV family, other extracellular structures have been described such as apoptotic bodies[6], exomeres that are likely not vesicular entities [7], and migrasomes [8] that might play a role in zebrafish embryonic development, and have been detected in human serum [9]. This review will mainly focus on exosomes and microvesicles.

The recent progresses within the EV community to deeply characterize the various subpopulations of extracellular vesicles has led to the establishment of a common nomenclature to distinguish EV subpopulations by their size (large and small EVs) or their origin (exosomes vs microvesicles). However, size, density and composition often overlap between EV subpopulations. It thus remains to be fully determined to what extent PM derived small (s)EVs differ from endosome derived EVs in composition or function. The respective contribution of exosomes and microvesicles to the total pool of EVs *in vivo* remains likewise an outstanding question (Box 1). Their high abundance in body fluids coupled to a proposed short half-life suggests sustained secretion *in vivo* [10]. Nonetheless, various reports suggest that EVs might be released in a constitutive as well as an induced manner [11,12] and could be cell-type dependent. Understanding EV biogenesis, uptake and function greatly benefited from *in vitro* and *in vivo* (mainly mice, but also *Drosophila* and *C. elegans*) studies [5,10]. However, live-visualization of EV secretion has long been impossible. This hampers the careful assessment of physiological release and uptake rates among cell types and tissues.

Consequently, a detailed analysis of EV half-life *in vivo* and careful evaluation of the relative contribution of different biogenesis- and release mechanisms lag behind. Recently developed approaches now allow visualization of EV release by single cells both *in vitro* [13,14] and *in vivo* [2,3], even though visualizing the release process *in vivo* itself remains a technically challenging task (Box 2). Concretely, tissue specific expression of an exosomal reporter, **CD63-pHluorin**, revealed that developing zebrafish embryos secrete massive amounts of exosomes in a syntenin-a dependent manner. In this context, the zebrafish model adds to the very few [15,16] *in vivo* studies of EV biogenesis pathways previously described *in vitro* [17]. These exosomes (majorly between 80-120nm in size) are secreted from the **yolk syncytial layer** (YSL) into the blood-stream, a conclusion further supported by *in situ* electron microscopic analysis of this layer. By exploiting tissue specific promoters in zebrafish, similar approaches could be used to determine physiological secretion rates and local spreading of exosomes and microvesicles for every tissue *in vivo* (Box 1, Figure 1a). **Mosaic expression** of markers like CD63-pHluorin will further allow close observation of EV behaviour directly after secretion, and will be useful to study the impact of the 3D *in vivo* tissue architecture on local EV retention or propagation. Interestingly, levels of circulating EVs increase with changes in physiological status [18,19] or during development of various pathologies [20–25]. EV secretion levels might thus be increased when homeostasis is disturbed and/or EV clearance is perturbed. These hypotheses could be now tackled using established zebrafish models of pathologies where EVs are already known to play a central role (cancer biology, cardiopathies, neuropathies, host-pathogen interactions, etc...) [26,27]. Overall, *in vivo* imaging of EV release will be instrumental to confirm and further explore EV-secreting tissues under physiological and pathological conditions (Box 3).

IN TRANSIT: DISSEMINATION AND BEHAVIOR IN BODY FLUIDS

In vivo imaging at high spatio-temporal resolution could reveal how EVs diffuse around secreting cells, how they cross biological barriers, and eventually how they reach the circulation before disseminating through the body (Figure 1b-c). For instance, spreading of zebrafish melanoma EVs can be tracked at various stages of metastasis *in vivo* using Syntenin2-GFP expressing melanoma cells [3]. *In vivo*, most cells are not in direct contact with the blood but rather with the interstitial fluid compartment that constitutes roughly three quarters of the total extracellular body fluid volume. Interestingly, high quantities of endogenous YSL-exosomes can be found in the interstitial fluid of zebrafish embryos [2]. This compartment might therefore be an overlooked reservoir of EVs, and might play an important role during homeostasis or pathology. Likely, it functions as a transit route for EVs toward blood vessels or lymphatic vessels, which have recently been shown to contain large amounts of EVs [28]. Alternatively, EVs could reach cells distant from blood vessels by other mechanisms, such as **transcytosis** or vascular leakiness (Figure 1b, Box1) [29–32]. Indeed, multiple *in vivo* studies suggest that EVs can cross cellular barriers [29,33], but the precise mechanisms involved largely remain to be determined. This is particularly relevant in cancer where vessels are known to be abnormally permeable.

In that context, zebrafish embryos offer a complex and stereotyped vascular network, suitable to the fine dissection of EVs behaviour in circulation and long-range dispersion. Fast imaging of EVs in the bloodstream reveals that, by contrast to large blood cells, EVs span on the entire width of the vessels (See Figure1). Notably, they also flow in close proximity to the vessel walls, where their velocities are reduced, according to the **Hagen-Poiseuille equation**, and where they can be found rolling on the surface of the endothelium [2,3]. This peculiar distribution is likely to impact the arrest and internalization of EVs that is further mediated by their surface adhesion molecules. Besides, we found that circulating EVs arrest more efficiently in venous regions than in arteries, suggesting that flow forces directly impact their ability to target specific vascular regions [3].

UPTAKE: ORGANOTROPISM, CELL IDENTITY AND MECHANISMS

In mice - where detection sensitivity is limiting - EVs injected in the blood circulation accumulate in a few specific organs (mostly spleen, liver and lungs) [34]. Furthermore,

specific organ targeting (and cell type specific targeting within these organs) was reported to depend on the integrin repertoire present at the surface of tumour EVs [35]. Further work is needed to describe how EV-loaded integrins mechanistically determine such specificity. This could be due to an organ-dependent enrichment of adhesion molecules such as integrin ligands in specific regions of the vasculature. Alternatively - or in addition - such organotropism could be explained by (i) the site of EVs injection, (ii) bloodstream features (ii) the capacity of EVs to escape from non-specific scavenging and/or (iv) the permeable nature of the target tissue. The size and transparency of the zebrafish embryo, where most organs are still being formed (Figure 1a), enables *in toto* screening that could provide a better understanding of EV biodistribution in real-time. In addition, high-resolution microscopy allows the identification of receiving cells and the dissection of uptake mechanisms. Interestingly, endogenously released exosomes *in vivo*, as well as pre-labelled tumour EVs subsequently injected in zebrafish embryos, are to a large proportion taken up by the **caudal vein plexus (CVP)** [2,3]. The CVP is highly enriched for scavenger endothelial cells (SEC), reminiscent of liver sinusoidal endothelial cells (LSECs) within the mammalian liver (Figure 1c). In addition, this vascular region displays slower hemodynamics, facilitating EV arrest [3]. Taking advantage of the high spatio-temporal resolution of the zebrafish model system, it was further demonstrated that the uptake by these endothelial cells is likely mediated by **scavenger receptors** in a dynamin-dependent manner, using a dynamin inhibitor and a competitive inhibitor for scavenger receptors both applied *in vivo* [2]. Of note, the interplay between receptors engaged on the target cell and specific surface EV proteins could lead to the direct activation of signalling pathways and might heavily impact the fate of these EVs. Indeed, subsets of EVs could have their primary function by conveying signals to receptors located at the PM of the target cell [36,37].

These studies likewise indicate efficient uptake of EVs by patrolling macrophages present in the lumen of blood vessels of the CVP. Such uptake is likely to occur via filopodia surfing and macropinocytosis (a dynamin-independent process, previously described *in vitro* [38]) and 'classical' endocytosis (Figure 1c). Genetic interference with receptor expression or modulation of their trafficking *in vivo* could provide more insight in these processes. Finally, disruption or saturation of clearance mechanisms due to sustained secretion levels of pathological EVs might expand the range of target cells [39], and thus "alter" the pathological role of these EVs. In this respect, approximation of physiological or pathological levels of EV secretion levels *in vivo* might prove to be pivotal for a true understanding of their significance. Live imaging at high resolution in zebrafish could thus help to further unravel cell type specific targeting of EVs over time, as well as the details of their dwelling time at the PM and the receptor(s) involved. In addition, it will allow a detailed analysis of their uptake and trafficking at subcellular level *in vivo*.

FATE: DEGRADATION, BACK-FUSION AND MESSAGE DELIVERY

Upon internalization, EVs follow three possible fates: (i) trafficking to lysosomes – leading to degradation/recycling of their content; (ii) back-fusion of EVs with the limiting membrane of late-endosomes - resulting in EV cargo delivery to the cytoplasm and/or integration of exosomal transmembrane protein into host cells membrane; (iii) re-secretion of EVs (transcytosis) (Figure 1c, Box1). Alternatively, EVs could fuse with the PM of the receiving cell to deliver cargo, but most reports indicate uptake before cargo delivery [40]. As the fate of EVs in the target cell will directly impact their function, live-imaging combined with Electron Microscopy can be a powerful tool to further decipher this aspect of EV physiology. *In vivo* imaging can be used to demonstrate trafficking to late-endosomal compartments by using EV markers tagged with a combination of pH-sensitive and non-pH sensitive markers (e.g. pHluorin-CD63-mScarlet [41]), in combination with pH-sensitive dyes, such as LysoTracker™ [3] or immuno-EM on previously identified recipient cell types/tissues by live-microscopy [2]. Functional cargo delivery *in vivo* can also be visualized using the Cre-stoplight system [42], or by loading EVs with other modulators of reporter genes. Interestingly, a recent report using ¹³C-labeled isotope labeling suggests that EV cargo that is (partially) degraded by lysosomes can be re-utilized by the cell [43]. Indeed, as suggested by accumulation of YSL-exosomes in the

endothelium following treatment with the lysosomal inhibitor Bafilomycin-A, *in situ* EM analysis showed the presence of EVs in degradative compartments [2]. In the context of zebrafish embryo development, uptake and lysosomal degradation of YSL-derived endogenous EVs by endothelial cells of the CVP were shown to provide trophic support [2]. Even so, trafficking to lysosomal compartments might not exclusively be linked to trophic support by degradation of EVs. Accumulation of internalized EVs in late endosomes/lysosomes of patrolling macrophages was demonstrated using correlative Light-Electron Microscopy (CLEM) [3]. Such approach can be efficiently performed in zebrafish and thereby unlock some of the remaining secrets related to functional cargo transfer to receiving cells. Despite their sorting to lysosomal compartments, tumor EVs could indeed induce macrophage activation, suggesting that transfer of EV message/content is likely to occur [3]. This phenotypic switch correlates with an increased metastatic outgrowth induced by tumor EVs [3]. Likewise, in a near future, the functional impact of EVs, extended to other biological questions, could be directly assessed in zebrafish by tracking phenotypic changes on receiving cells after EV uptake and modulation thereof [2,3].

CONCLUDING REMARKS

Despite major recent advances, the exact relevance of exosomes and other EVs *in vivo* remains obscure [44]. Moreover, while pathologies, and especially cancer, are widely studied, their exact function in normal physiology remains elusive. In our view, *in vivo* approaches compatible with high spatio-temporal resolution imaging, now possible in zebrafish embryos (and hopefully adults in the future), will be instrumental to unlock fundamental aspects of the journey and functions of EVs.

Text Box 1 | Fishing for answers: outstanding questions in the EV field

As direct observation of EVs is cumbersome in rodent models, demonstration of their roles in a fully physiological *in vivo* context is still lacking [44]. Multiple questions thus remain unanswered: How often are EVs secreted, and what are the triggers? Once secreted, do they travel long-range distances or stay in the vicinity of the secreting cell, and why? Are they able to overcome biological barriers and what is their half-life? What part of EV physiology eventuates extracellularly without requiring internalization, or even cell-independently? How often and how many EVs need to reach a target cell to elicit a response? What proportion needs to be internalized or is degraded? Addressing these questions requires model systems that allow for resolute, dynamic observation of EV release, trafficking and uptake, as well as tools to modulate EV release and targeting. Rodent models are not yet adapted to such tasks, and invertebrate models, despite other advantages, mostly lack circulatory systems similar to human. Zebrafish embryos, by contrast, offer many advantages, including rapid development, low costs, and amenability to genetic modification, natural transparency – allowing non-invasive imaging - and vascular networks. They successfully allow probing single EVs at high spatio-temporal resolution by combining high-speed *in vivo* microscopy with resolute electron microscopy.

Text Box 2 | Technical aspects of EV imaging *in vivo*

Recent studies demonstrate that imaging of single EVs *in vivo* is feasible, at a level that it can be used to investigate biological processes [2,3]. Even though the majority of the EVs that were studied (50-150nm) are below the resolution limit (~220nm), they can be easily detected provided they are bright enough. Temporal resolution of imaging remains an issue. Our studies used injection of pre-labelled EVs in combination with a confocal microscope [3] as well as tissue specific expression of a genetically encoded CD63-pHluorin in combination with a spinning disk microscope [2]. The detection limit for EVs ultimately depends on multiple aspects, including the (concentration of the) fluorophore, the size of the EV, the imaging speed and depth, the background fluorescence level and the suitability of the microscope set-up. In case of genetically encoded EV markers, such as fluorophore-tagged tetraspanins, mosaic expression and/or the use of pH sensitive markers can successfully be

used minimize the background signal coming from expressing cells that would otherwise mask the relatively low signals coming from EVs [2,14]. The suitability of the microscope setup, finally, will be determined by the numerical aperture of the objective, laser wavelength and intensity, and the sensitivity of the camera. Finally, the fact that extracellular (non-endocytosed) EVs are motile (i.e. move with Brownian motion in absence of flow, or take the speed of the flow) adds temporal restrictions to the exposure time. In addition to *in vivo* photonic imaging, EVs can be observed by electron microscopy in fixed samples. *In situ* immunolabeling can further discriminate between different EVs subtypes or identify tagged EVs [2], while correlation between light and electron microscopy (CLEM) offers the possibility to combine *in vivo* tracking of EVs with their high resolution imaging [3]. Finally, super-resolution microscopy will in some specific cases provide improved z- and lateral resolution to refine intracellular trafficking of EVs in producing and receiving cells [45].

Text Box 3 | Pathological models in ZF

Zebrafish can be used to recapitulate various aspects of human pathologies. While sharing less homology with humans than rodents, 71.4% of human genes and 82% of human genes involved in diseases have at least one zebrafish orthologue [46]. This opens the possibility for genetic cancer models for brain tumors, including glioma, but also for melanoma, malignant peripheral nerve sheath tumors, leukemia (T-ALL, B-ALL, AML), thyroid cancer, colon adenoma and hepatocellular carcinoma, pancreatic cancer, rhabdomyosarcoma [47,48]. This also includes the possibility of xenografting human cancer cells [49,50], in embryos or in immunodeficient adults [51], using zebrafish as real-time ‘avatar’ to predict individual patient’s tumor responses to therapy, opening the door to personalized medicine [48]. Apart from cancer, zebrafish can also be used as a model system to study host–pathogen interactions [52], including the possibility to study the functionality of Outer Membrane Vesicles in zebrafish [53], cardio-vascular pathologies [54], gastrointestinal diseases [55], kidney diseases [56], muscular diseases [57], metabolic disorders [58] and neurological disorders [59], including Alzheimer’s disease [60]. In most of these pathologies, EVs are suspected to contribute to disease progression, but their direct contribution *in vivo* remains to be determined.

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Figure Legends

Figure 1: Zebrafish, a new model to unlock the secrets of EVs *in vivo*. Multiple questions surrounding EV biology could be addressed using zebrafish embryos. A) Schematic depiction of a 48hpf zebrafish embryo indicating the organs that are being formed. B) EV secretion and dispersion can be analysed in zebrafish embryo. The electron microscopy picture (Verweij et al. Dev Cell 2019) illustrates the release of extracellular vesicles (pointed by black arrows) from the Yolk Syncytial Layer (YSL) to the blood circulation (indicated by (1) and a dashed box in the embryo scheme). Below, the scheme shows the processes that can be studied by tracking labelled exogenous (in blue) and endogenous EVs (eosomes in green; microvesicles in red) at single EV resolution, including secretion and local dispersion. C) Zebrafish embryo can be used to study distant uptake and fate of labelled EVs. The picture is taken from live confocal microscopy in the Caudal Vein Plexus (CVP), (indicated by (2) and a dashed box in the embryo scheme) of transgenic embryos expressing a red endothelium and green macrophages injected with Cyan fluorescent EVs (Hyenne V. unpublished). Similar to (B), the scheme below illustrates the events that can be analysed *in vivo*.

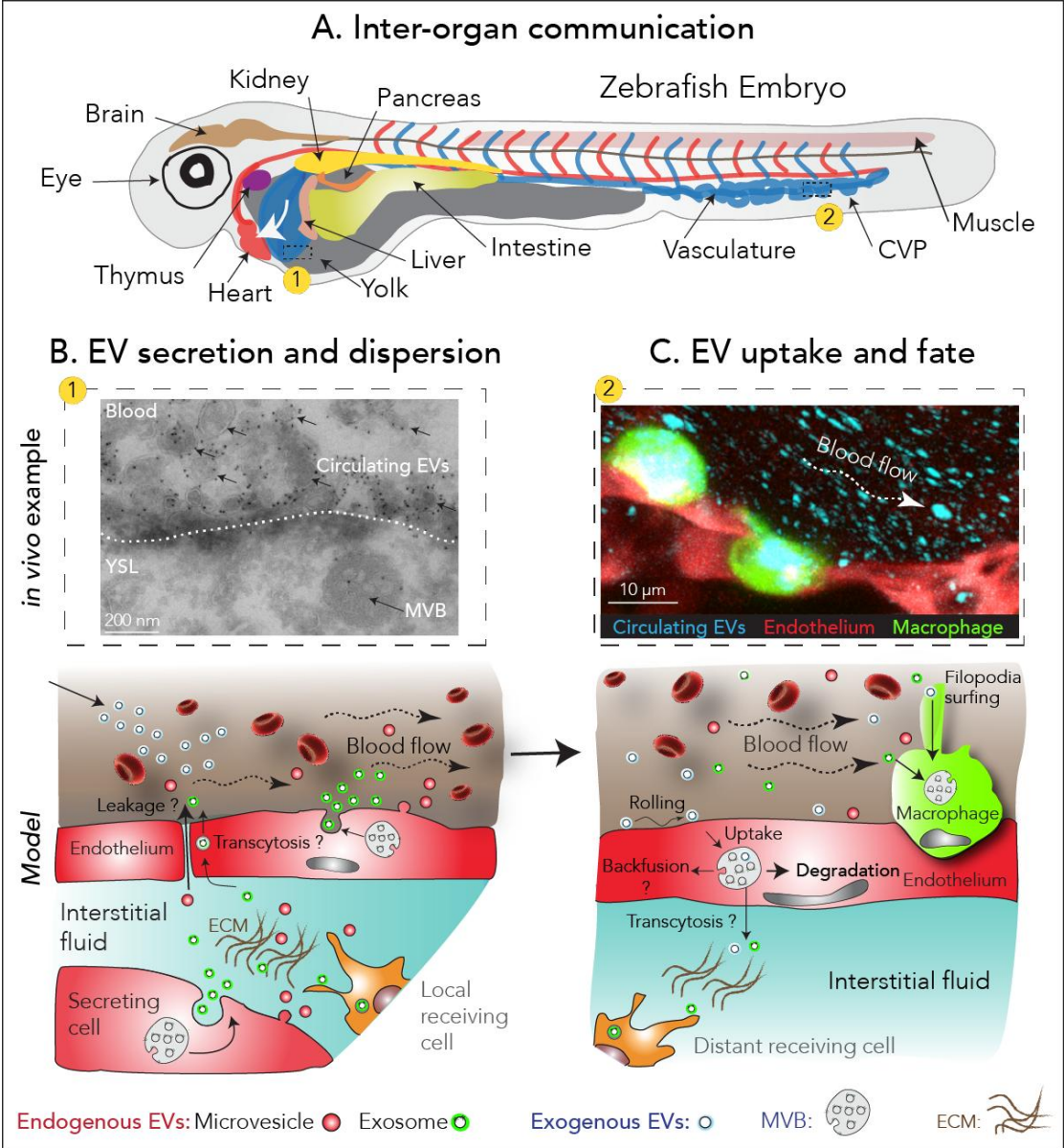


Figure 1