Short Report

Title**: Engineered *Salmonella* allows real-time heterologous gene expression monitoring within infected zebrafish embryos.**

Authors: Carlos Medina, Eduardo Santero, Jose Luis Gómez-Skarmeta & Jose Luis Royo*.

Affiliations:
Centro Andaluz de Biología del Desarrollo. Universidad Pablo de Olavide/Consejo Superior de Investigaciones Científicas/Junta de Andalucía. Ctra. Utrera, Km 1. 41013-Sevilla, Spain.

*to whom correspondence should be addressed:
jlroysan@upo.es
+34 954977445

Abstract:

Microbial host-pathogen interactions have been traditionally well studied at genetic and physiological levels, but cell-resolution analyses have been particularly scarce. This has been especially remarkable for intracellular parasites for two major reasons: first, the inherent loss of bacteria traceability once infects its hosts; second and more important, the limited availability of genetic tools that allow a tight regulated expression of bacterial virulence genes once inside the host tissues. Here we present novel data supporting the use of zebrafish embryos to monitor *Salmonella enterica* serovar Thyphimurium infection. Intravenous infection of *Salmonella* can be easily monitored using *in vivo* fluorescence that allows the visualization of free-swimming bacteria through the circulatory system. Moreover, we have engineered *Salmonella* to voluntarily activate heterologous gene expression at any point during infection once inside the zebrafish macrophages using a salicylate-based expression system. This approach allows real-time cell-resolution *in vivo* monitoring of the infection. All together, this approach paves the road to cell-based resolution experiments that would be harder to mimic in other vertebrate infection models.

Keywords: In vivo protein expression; Salmonella pathogenesis; Zebrafish;

Among the different bacterial pathogens, *Salmonella* spp takes importance since it can induce gastroenteritis, enteric fever or bacteraemia that constitute a significant economic loss of productive work-time (Arnold et al., 2007). Besides the acute health problem, some studies have demonstrated that *Salmonella* infection increases the risk of developing inflammatory bowel diseases and other gastrointestinal disorders, including chronic inflammation and bladder cancer (Bell, 2010). Although many infection-related genes have been identified as virulence factors, functional studies addressing the role of the different genes in the distinct infection steps remain scarce. During the past years, murine models of infectious diseases have been the host of choice for analyzing host-pathogen interactions during infection (Duan et al., 2009). Unfortunately, once *Salmonella* is inside the mouse, the cellular resolution level is abolished (Liu et al., 2010). An alternative approach to study *Salmonella* infection has relied on monolayer cell culture systems (Lahti et al., 2009) or 3D reconstructions of tissues which include polarized intestinal cell culture models (Barrila et al., 2010), ileal loop models or spheroids (Kasinskas and Forbes, 2007). However, studying the interaction between the bacteria and the immune system requires a whole-organism infection.

The emergence of the zebrafish (*Danio rerio*) as infectious disease model has provided new information and insights into pathogenesis studies. The innate immune system of the zebrafish embryo starts developing during the first day post-fertilization (dpf). Differentiated myeloid cells invade the head mesenchyme tissue or join the blood circulation (Herbomel et al., 2001) showing specific adherence to other parasites such as mycobacteria injected into the blood which are rapidly phagocytised (Davis et al., 2002). They are also able to sense the presence of bacteria injected into one of the closed body cavities and to respond by migration to the infection site (Herbomel et al., 1999). Therefore, the zebrafish embryo model is useful for determining the role of innate immunity in responses to different infectious agents, as it is uncoupled from adaptive immunity (van der Sar et al., 2006).

To date, some examples have highlighted the potential of zebrafish for monitoring infections, given its transparency and rapid development (van der Sar et al., 2003). Here we go one step forward, focusing our attention to the control of gene expression once the bacteria reside in the host. We have engineered the *Salmonella* with a salicylate-based expression system. Salicylate is innocuous to the zebrafish at the given concentrations and diffuses across the different embryonic tissues to reach the intra-bacterial concentration necessary to trigger heterologous expression inside the macrophages. Here we present a proof of concept and demonstrate that we can voluntarily activate heterologous gene expression of *Salmonella* once inside the zebrafish host.

Strain, plasmid and growth conditions: Bacterial strains used in this work were grown aerobically at 180 rpm and 37ºC in LB medium and supplemented when necessary with ampicillin (100 μg/ml). *S. Typhimurium* MPO94 (14028Δ*trg:: nahR*/P_{sal}–*xylS2*/P_{tac}–*gfp)*, which contains the regulatory module, has been previously described (Medina et al., 2011) The regulatory module combines *nahR* with *xylS2* under the control of the NahR-responsive P_{sal} promoter integrated into *Salmonella* chromosome. The reporter gene is inserted into a separate expression module downstream of the XylS2-responsive P_m promoter, in the reporter plasmid pMPO1047 (Ap^R), a pSC101-based expression vector containing a rrnBT1T2-Pm::MCSII-dTomato fusion, translated from an optimized T7 Shine-Dalgarno sequence**.** The binding of salicylate to NahR and XylS2 activates the expression of genes controlled by the P_{sal} and P_m promoters respectively (Royo et al., 2007).

Zebrafish care and embryo microinjection: Zebrafish were obtained and maintained from our breeding colony under standard conditions according to previously established procedures (http://zfin.org). Embryos for injection were obtained from crosses of wild-type AB/Tuebingen zebrafish. Fertilized eggs were kept at 28 °C in Petri dishes with 20 ml of aquarium water with 0.003% 1-phenyl-2-thiourea to prevent pigmentation. Microinjection needles were manufactured from 1 mm diameter glass capillary (inner diameter 0.58 mm) (A-M Systems Inc, Carlsborg, WA). Capillars where sharpened using a Suttere P97 puller according to manufacturer instructions.

Infection and heterologous gene expression: 30 hours post fertilization (hpf) embryos where anesthetized with 0.02% tricaine and disposed on an agarose array. Needles where loaded with a bacterial suspension of ≈$5x10^2$ cfu/nl. 0.25% Congo red was used as an injection tracer. Injection was performed in the axial vein using a Narishige miroinjector under conventional binocular. For each condition 50-100 embryos where injected. Infected zebrafish were kept at 28 °C in Petri dishes with 20 ml of E3 medium with 0.003% 1-phenyl-2-thiourea to prevent melanogenesis. For induction, 2 mM sodium salicylate was added to the Petri dish. Embryos were monitored under fluorescence binocular for 48h.

Primary cell culture: Infected embryos where anesthetized and resuspended in 1ml phosphate-buffered saline containing 0.25% trypsin and incubated at 30ºC for 15 min. Cells where homogenized with gentle pippetting and deposited on Petri dishes with DMEM supplemented with 10% foetal bovine serum and 100 μg/ml gentamicin, which precluded de novo re-infection while culturing. Cells where let to attach for 30 min at 30ºC. Supernatant was aspired and replaced by new medium.

Live imaging: For zebrafish live imaging, embryos were anesthetized and placed under an inverted fluorescence microscope (Leica Systems). Excitation of filters used where λ=546/12 nm for Tomato fluorescence, and λ=470/40 nm for GFP. Pictures and video where taken with LAS Multi-Time Module and further edited with Image J freeware and iMovie 7.1.4 (585) software, respectively.

The MPO94 strain containing the GFP constitutively expressed from the bacterial chromosome under the control of P_{tac} promoter has been previously reported to track *Salmonella* during infection of HeLa cells (Medina et al., 2011). In order to assay the *in vivo* visualization of infection, we injected the axial vein of 30 hpf zebrafish embryos with $\approx 10^3$ cfu, as stated above. Successful injections were visible when Congo red staining perfunded the blood stream. A few minutes upon injection we could easily detect free bacteria in the circulatory system. This was particular evident in the caudal vein, the dorsal aorta and other capillars irrigating the head (Figure 1 A-D, Supp Video 1). Between 2 to 5 hours post infection (hpi), most freely swimming bacteria were phagocyted. This was observed while either circulating through the caudal vein or immobilized in other peripheral vessels. Under fluorescence microscope, 10-20 of these macrophages containing bacteria where visualized and recorded 24 hpi (Figure 1 E-G).

Next, we wanted to assay whether we could trigger heterologous expression in those bacteria once they lie inside macrophages. Initial characterization of the salycilate-dependent expression system in bacterial cultures indicated that maximum activation of the expression system was obtained with 2-4 mM salycilate. This inducer concentration was found to be completely innocuous to the zebrafish embryos, and did not affect their development or behaviour (data not shown). Thus, a strain of *Salmonella* containing the GFP constitutively expressed from the chromosome, and the dTomato fluorescent protein in a plasmid under the control of the P_m promoter, was used to infect the zebrafish embryos. After parenteral administration, zebrafish where incubated at 28ºC and the infection was allowed to proceed. When phagocytosis was detected, embryos where split in two plates containing E3 media with or without 2 mM salycilate. After 4h, we could detect *de novo* dTomato expression inside the phagocyted bacteria under induced conditions (Figure 2). This demonstrates that the intracellular concentration of salycilate in the macrophages was sufficient to trigger heterologous bacterial gene expression once inside the cell cytoplasm.

Finally, we assayed whether infected macrophages could be isolated to perform further single-cell resolution studies. To that end infected 48 hpf embryos where sacrificed and cell suspensions were generated. Cell suspensions where incubated in Petri dishes with DMEM medium at 30ºC as described above. After 1h, the remaining medium was aspirated and replaced by new one and

examined under microscope. We could detect a high percentage of cells attached to the cell surface, some of them harbouring GFP+ *Salmonella* inside them (Figure 3). Thus, using this procedure, infection can be monitored *ex vivo* by following *Salmonella* replication and the final death of the eukaryotic cell.

Altogether, here we present novel data supporting the use of zebrafish embryos to monitor *Salmonella thyphimurium* infection. Intravenous administration of *Salmonella* can be easily monitored using *in vivo* fluorescence that allows the visualization of free-swimming bacteria throughout the circulatory system. However, to date no regulated expression have been tested in intracellular parasites using zebrafish as host. Most systems used in biotechnology are not suitable for studies involving bacterial behaviour inside mammalian cells. In fact, several *in vivo* prokaryotic inducible expression systems used that respond to external stimuli, such as the tetracycline responsive bacterial tetracycline repressor, are limited for studying host-pathogen interactions because of its toxicity, inducer side effects or promoter leakiness. Here we have demonstrated that we can trigger heterologous expression from the bacterial P_m promoter once it lies inside the macrophages when salycilate is dispensed in the aquarium water. We propose that this approach could be a valuable tool for future experiments, paving the road to cell-based experiments that would be hard to mimic in mice.

ACKNOWLEDGEMENTS

We have to thank Yolanda Roncero and Carmen Hidalgo for zebrafish handle and embryo source. This work was funded by grants BFU2010-14839, CSD2007-00008, CSD2007-00005 and Proyectos de Excelencia CVI-3488 and P07-CVI-02518 from the Spanish and Andalusian Governments, respectively. Jose Luis Royo holds a JAE DOC contract from the Spanish National Research Council (CSIC).

Figure legends:

Figure 1: *Salmonella* detection by microscopy on infected zebrafish. *Salmonella* was injected in the axial vein (A) as described and visualized in the bloodstream early post infection (B, C, D), and inside macrophages 24 hpi (E; 100X, F; 320X, G; 640X).

Figure 2: Inducible intracellular expression of dTomato protein. Fishes were injected with *Salmonella* MPO94 harbouring pMPO1047 plasmid. Infection was allowed to proceed prior to split them on plates containing (E-I) or not (A-D) salicylate. Left to right panels show phase contrast, constitutively expressed GFP, salicylate-induced dTomato protein and a 640X magnification of bacteria inside macrophages.

Figure 3: Macrophages isolated from fishes. Fishes were sacrificed and a cell suspension was prepared by trypsin treatment to obtain isolated macrophages from plates containing (A-D) or not (E-H) salicylate. Left to right panels show phase contrast, constitutively expressed GFP, salicylate-induced dTomato protein and merged images.

REFERENCES

Arnold, D.L., Jackson, R.W., Waterfield, N.R. and Mansfield, J.W., 2007. Evolution of microbial virulence: the benefits of stress. Trends Genet 23 (6), 293-300.

Barrila, J.et al., 2010. Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. Nat Rev Microbiol 8 (11), 791-801.

Bell, G., 2010. Fluctuating selection: the perpetual renewal of adaptation in variable environments. Philos Trans R Soc Lond B Biol Sci 365 (1537), 87-97.

Davis, J.M.et al., 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17 (6), 693-702.

Duan, Y.et al., 2009. Complete genome sequence of citrus huanglongbing bacterium, 'Candidatus Liberibacter asiaticus' obtained through metagenomics. Mol Plant Microbe Interact 22 (8), 1011-1020.

Herbomel, P., Thisse, B. and Thisse, C., 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126 (17), 3735-3745.

Herbomel, P., Thisse, B. and Thisse, C., 2001. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol 238 (2), 274-288.

Kasinskas, R.W. and Forbes, N.S., 2007. Salmonella typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis. Cancer Res 67 (7), 3201-3209.

Lahti, D.C.et al., 2009. Relaxed selection in the wild. Trends Ecol Evol 24 (9), 487-496.

Liu, X., Lu, R., Xia, Y. and Sun, J., 2010. Global analysis of the eukaryotic pathways and networks regulated by Salmonella typhimurium in mouse intestinal infection in vivo. BMC Genomics 11 722.

Medina, C. et al., 2011. Improved expression systems for regulated expression in Salmonella infecting eukaryotic cells. PLoS One 6 (8), e23055.

Royo, J.L.et al., 2007. In vivo gene regulation in Salmonella spp. by a salicylate-dependent control circuit. Nat Methods 4 (11), 937-942.

van der Sar, A.M.et al., 2003. Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections. Cell Microbiol 5 (9), 601-611.

van der Sar, A.M.et al., 2006. MyD88 innate immune function in a zebrafish embryo infection model. Infect Immun 74 (4), 2436-2441.

Figure1

Click here to download high resolution image

Figure 1

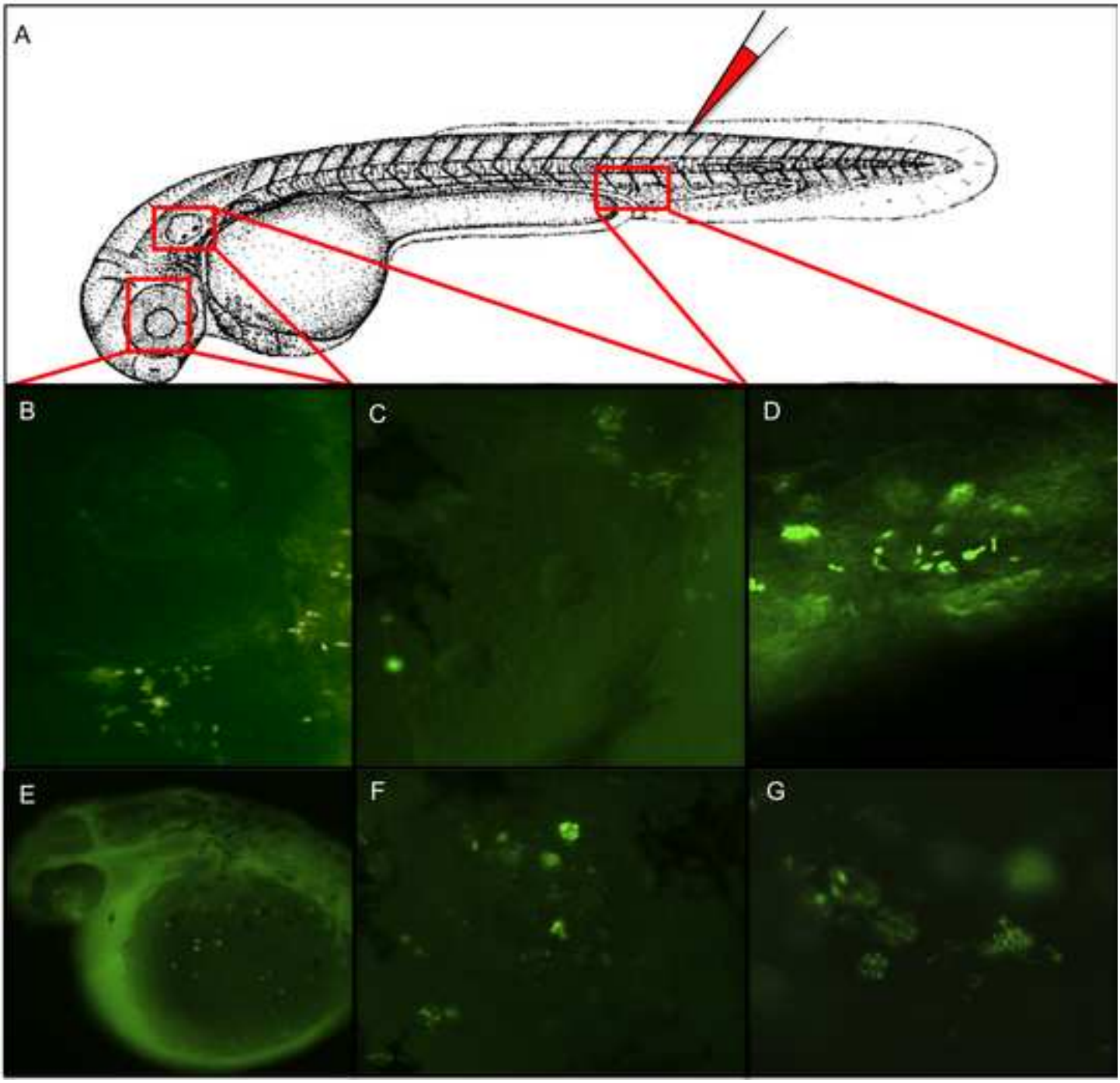


Figure2
Click here to download high resolution image

Figure 2

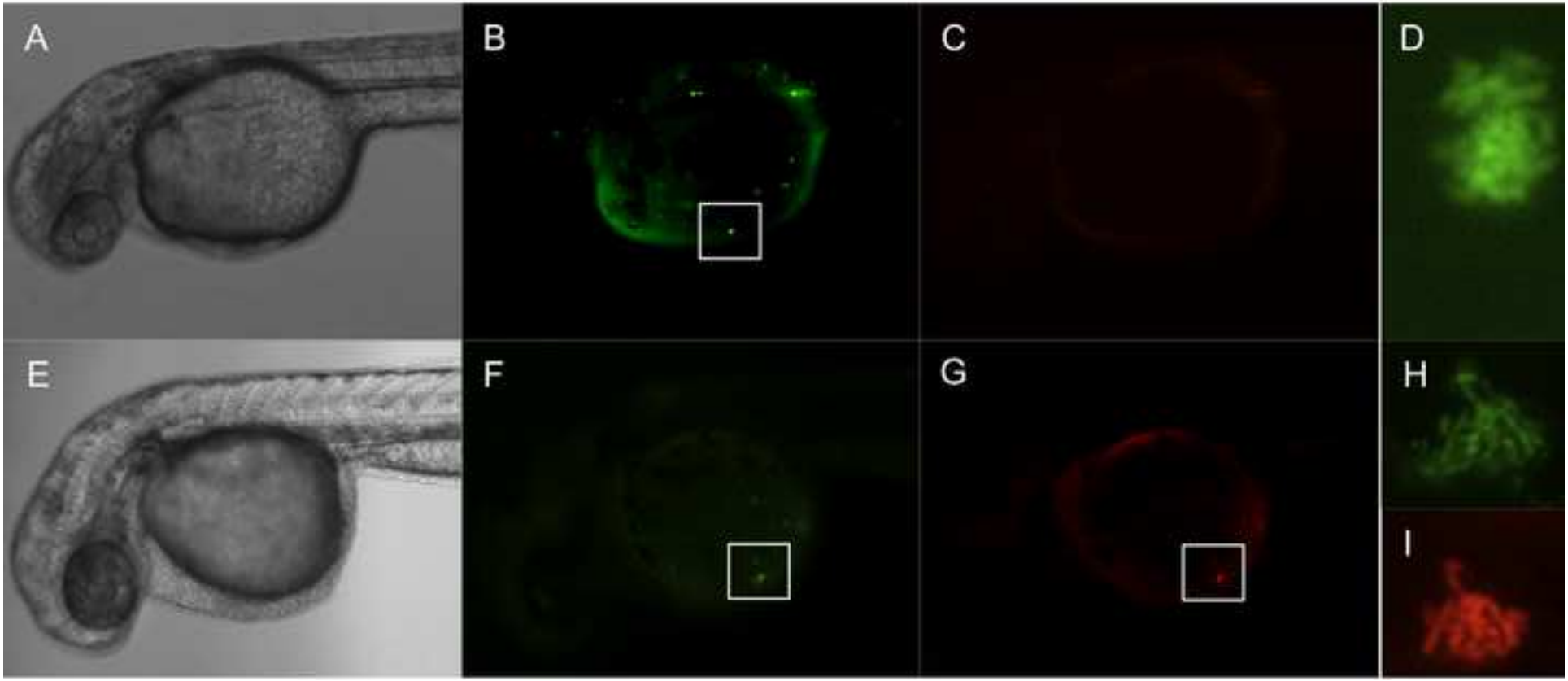
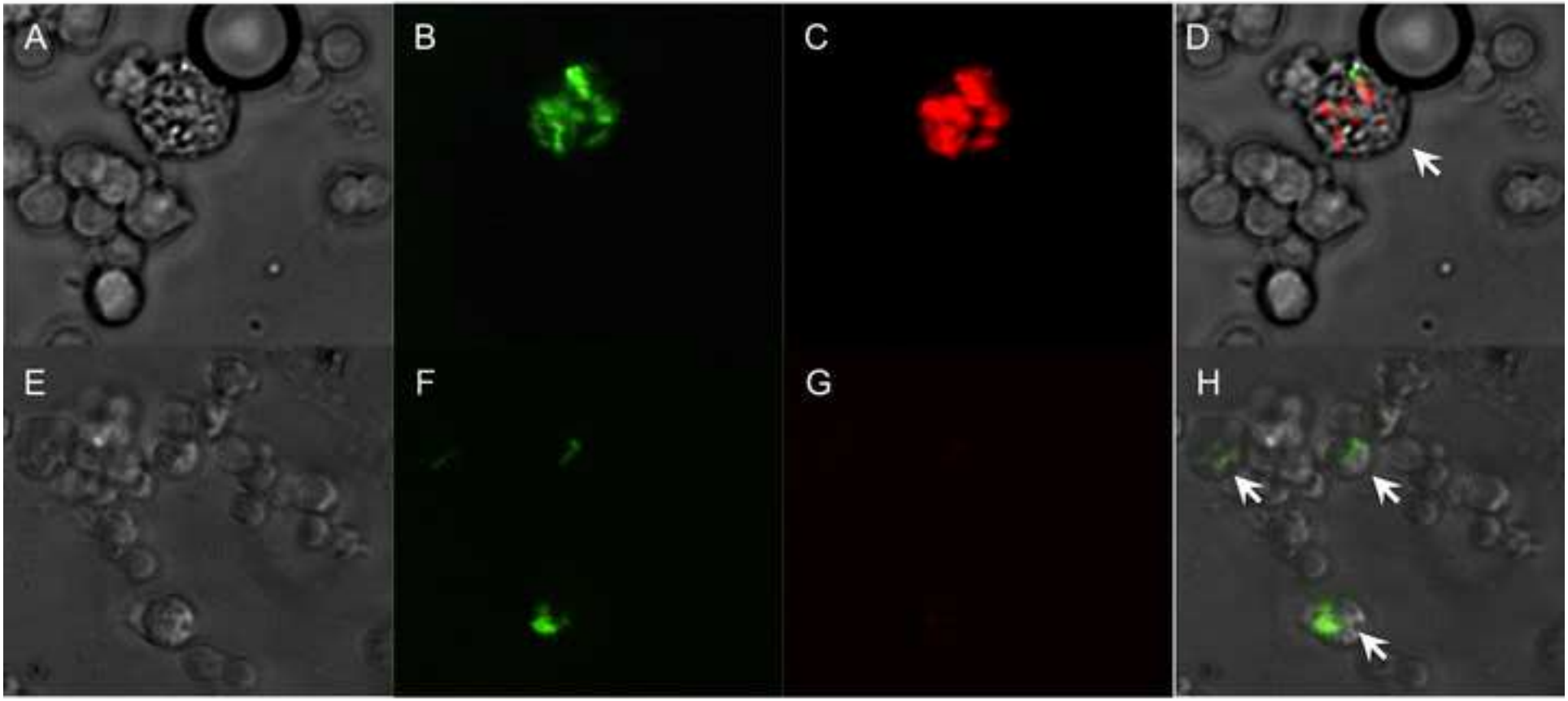


Figure3

Click here to download high resolution image

Figure 3



Supplementary Files

Click here to download Video Still: Suplementary video.avi