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Coupling clearing and hybridization chain reaction approaches to investigate gene expression in organs inside intact insect heads

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Abstract

Detecting RNA molecules within their natural environment inside intact arthropods has long been challenging, particularly in small organisms covered by a tanned and pigmented cuticle. Here, we have developed a methodology that enables high-resolution analysis of the spatial distribution of transcripts of interest without having to dissect tiny organs or tissues, thereby preserving their integrity. We have combined an in situ amplification approach based on hybridization chain reaction, which enhances the signal-to-noise ratio, and a clearing approach that allows the visualization of inner organs beneath the cuticle. We have implemented this methodology for the first time in Hemiptera, mapping two salivary aphid (*Acyrtosiphon pisum*) transcripts, the effector c002 and the salivary sheath protein SHP. With a multiplex approach, we could simultaneously detect different mRNAs in mounted pea aphid head-thorax samples and show that they were distributed in distinct secretory cells of salivary glands.

Research Highlights

- Combining hybridisation chain reaction and clearing allows the detection of transcripts in intact aphids heads.
- The transcripts of the two salivary proteins c002 and SHP are compartmentalized in distinct secretory cells of the principal glands.

KEYWORDS

aphids, clearing, cuticle, hemipteran, hybridization chain reaction

1 | INTRODUCTION

The study of gene expression by RNAseq approaches has been extensively used to characterize multiple biological processes (Stark et al., 2019). More recently, the possibility of moving from tissue to cell-level gene expression analysis has confirmed the high level of

gene expression heterogeneity in any given tissue (Jovic et al., 2022).

Both bulk and single-cell RNAseq transcriptomics analyses start with destroying the tissue structure and extracting RNA to be quantified. The spatial information in the intact tissue is thus lost with these approaches. Once genes of interest are identified, the possibility of visualizing their expression in the context of the tissues at a cellular level remains paramount to understanding the analyzed processes, tissue organization, and function within an organism. Two-dimensional

Stefano Colella and Marilyne Uzest contributed equally to this work.

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approaches using histology samples have been extensively used, and more recently, three-dimensional (3D) techniques have been developed to monitor RNA and protein expression in tissues at the single-cell level.

The possibility of visualizing the expression of genes of interest in 3D in intact or semi-intact organisms brings more detailed information that can be essential to characterize the biological processes under study. Using such techniques on arthropods is particularly challenging due to the cuticle's intrinsic properties, including lack of transparency, autofluorescence, and low chemical permeability, thus impeding the labeling of target molecules.

Among arthropods, aphids are phloem sap-feeding hemipterans that constitute a major agricultural pest and are vectors of around 30% of known plant viruses (Brault et al., 2010). Working on aphids, we face cuticle challenges and small size, limiting the use of classic histology for some tiny organs, resulting in the need for a high number of histological serial sections to be generated to visualize the targeted tissues of interest. Several clearing methods have been developed in the last decade to allow volume imaging and exploration of organs of fixed organisms in their natural environment (Molbay et al., 2021; Vieites-Prado & Renier, 2021).

In situ hybridization to map mRNA expression has been extensively used to study different biological processes. However, multiplexing, that is, visualizing multiple mRNAs simultaneously, has long been challenging in several organisms. The hybridization chain reaction (HCR) method uses the self-assembly property of DNA to amplify the signal and thus allow the detection of target molecules at high resolution. Since its initial introduction (Dirks & Pierce, 2004), HCR has been further developed and optimized (Choi et al., 2014, 2018; Raj et al., 2008). HCR has been tested and validated on various organisms (Choi et al., 2016), including among arthropods fruit fly embryos, and it has been recently applied to detect mRNA on whole-mount mosquito antennae (Herre et al., 2022).

In this study, we have developed a new methodology to visualize multiple mRNA transcripts beneath the cuticle within intact heads of the hemipteran aphid species *Acyrtosiphon pisum*. To clear the aphid sample, we chose the immunolabeling-enabled 3D imaging of solvent-cleared organs (iDISCO+) method (Renier et al., 2014, 2016). This clearing approach had already been used on adult mosquitoes (De Niz et al., 2020) but has never been implemented on hemipterans nymphs or adults. We coupled this clearing approach with the HCR RNA fluorescent in situ hybridization (RNA-FISH) method to detect mRNA expression in salivary glands inside the aphid's head. As we explored a novel technology combination on the pea aphid, we chose two genes previously identified as expressed in the salivary glands, the effector c002 (ACYPI008617; XM_001948323) (Mutti et al., 2006, 2008) and the salivary sheath protein SHP (ACYPI009881; XM_001943863) (Carolan et al., 2009, 2011; Rao et al., 2013). Aphid salivary glands include two principal salivary bilobed glands (psg) and two accessory salivary glands (asg) (Ponsen, 1972; Weber, 1928). They have been thoroughly described by microscopy-based anatomical observations (Ponsen, 1972; Weber, 1928; Witlaczil, 1882), which allows data analyses in light of an established anatomical description.

2 | MATERIALS AND METHODS

2.1 | Aphid rearing

In this study, we used an aphid colony of *Acyrtosiphon pisum* Harris (clone LL01) maintained on faba bean (*Vicia faba* cv Robin Hood) in an environmental growth chamber at 18°C with a 16/8 h day/night photoperiod.

2.2 | Collection and fixation of aphid tissues

Aphid adults were collected and cold anesthetized in a chilled petri dish on ice. Abdomens were severed and discarded from the insects using a G26 needle under a dissecting microscope. The head-thorax parts were kept in RNase-free phosphate-buffered saline buffer [4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.3, PBS] during sample collection. They were then transferred for fixation in a 2 mL glass tube (Wheaton V vial) containing freshly prepared PBST buffer (PBS supplemented with 0.1% Tween 20) and 4% paraformaldehyde (15,713, EMS). The fixative was incubated at 80°C for 20 min to depolymerize the solution as much as possible, prior to use. Samples were incubated for 45 min at room temperature with gentle shaking. After three rinses in PBST, the samples were dehydrated in a graded methanol series (30%, 50%, 70%, 100%, and 100%) for 10 min at room temperature for each solution. Head-thorax samples, dehydrated in methanol 100%, were stored at -20°C until use for HCR labeling and clearing.

2.3 | Hybridisation chain reaction RNA fluorescent in situ hybridisation, HCR RNA-FISH

Four pairs of probes were designed for each target gene using HCR probe 3.0 Maker (https://github.com/rwnull/insitu_probe_generator). The probes and amplifiers used are listed in Table S1.

All buffers for probe hybridization, probe wash, and amplification were provided by Molecular Instrument (Los Angeles, CA, USA). We followed the protocol for HCR RNA FISH originally developed for whole-mount fruit fly embryos (Choi et al., 2016) (see Supplementary Materials and Methods S1 for more details). After fixation and hybridization, samples were mounted into droplets of 1% agarose (LE-8200, Euromedex, Souffelweyersheim, France) in a handmade incubation chamber to facilitate handing orientation of aphid specimens for laser scanning confocal microscopy (LSCM) observation (Figure S1).

2.4 | Tissue clearing

Samples were cleared using the iDISCO+ approach (Renier et al., 2016). Samples were transferred in small glass bottles (4 mL) and dehydrated in a graded methanol series (from 30% to 100%) for 10 min per wash. They were incubated once in 2.25 mL of fresh methanol-dichloromethane (1:3 v/v) solution for 3 h at room temperature under gentle shaking and

twice 15 min in 1 mL dichloromethane (270997, Merck KGAA, Darmstadt, Germany). They were then washed with 1 mL in dibenzylether (A18447, Thermo Scientific, Waltham, MA, USA) and transferred in a 2 mL vial polypropylene tube with a screw cap. Samples were incubated three times with 1 mL dibenzylether for 15 min at room temperature. Finally, the tubes were filled with dibenzylether to prevent oxidation and stored in a dark box at room temperature.

2.5 | Mounting and imaging of aphid salivary glands

A blade single edge carbon steel (71960, Electron Microscopy Science, Hatfield, PA, USA) was fixed with Loctite glue on a microscope glass slide. Samples in agarose were placed in the hole in the blade's center prefilled with dibenzylether. A second drop of dibenzylether was added, and a coverslip was placed to close this incubating chamber that was sealed with Epoxy (Loctite Colle Power Epoxy, Henkel, Düsseldorf, Germany) (Figure S1). After overnight drying, samples can be immediately observed and kept for further observations up to 2 months. Aphid head-thorax parts were imaged with a Zeiss LSM 900 (Carl Zeiss AG, Oberkochen, Germany) inverted confocal microscope with a 10 \times objective. For laser scanning imaging, the parameters used were as follows: image size 4365 \times 4365, average 2, speed 0.48 μ s/pixel, bidirectional scan. To observe the insect morphology and the detailed anatomy of internal organs, lasers used were 405 nm, 488 nm with Multialkali detector. To detect the amplifiers, lasers used were 561 nm, 640 nm with GaAsP-PMT detector.

2.6 | Image processing and analyses

Image processing was performed with Zen blue software (Carl Zeiss AG, Oberkochen, Germany) for light transmission microscopy with DIC extended depth focus. All LSCM images were processed with the LSM plus software of Zen Blue. Maximum projections of confocal z-stacks were carried out in Fiji Image J (Schindelin et al., 2012). The main problem at this stage is that the observed samples can never be oriented in the same position. Ensuring equivalent laser power across the observed object is essential, and the variable sample orientation makes this extremely difficult. It was, therefore, mandatory to adjust the laser intensity using an internal control present in the aphid heads. The central nervous system was used to normalize the parameters in all the imaging experiments. The autofluorescence signal for Alexa 546 and Alexa 647 was measured on the central nervous system, and the minimum brightness was adjusted accordingly, thus optimizing the imaging process. The co-localization analysis was performed with DiANA (Distance Analysis plugin for Fiji enabling 3D segmentation for object extraction) (Gilles et al., 2017). DiANA was used to individually segment the fluorescent objects for each probe in the images acquired and overlapping objects. The volume of each object was calculated. To illustrate the results, we recorded a video of the 3D reconstruction using Image J's segmentation editor and 3D viewer plugins (see Movie S1).

3 | RESULTS

3.1 | Clearing allows the visualization of internal organs through intact aphid head-thorax samples

We first evaluated the efficiency of the solvent-based iDISCO+ clearing protocol with unlabeled aphid heads. The treatment produced excellent results, with the treated specimens showing significantly higher transparency than that of untreated ones. The difference is evident when comparing the widefield images of uncleared aphids (Figure 1, panels a–f) and the ones on the treated samples (Figure 1, panels g–l). The protocol's success in allowing the visualization of multiple organs inside intact aphid specimens is a key step to identify specific transcripts and proteins in their original anatomical localization. These results allow working on intact head-thorax samples, thus preserving organ structures and untouched cells that can be partially disrupted when using dissection approaches. The images corresponding to optical sections from the ventral to the dorsal side (Figure 1) show that internal organs can be visualized only in the cleared samples. We can, for example, distinguish both types of salivary glands. We can also visualize the lamina (L) and medulla (M) optic lobes brain sections (Figure 1j) previously characterized in aphids using in situ sections analyses and 3D reconstruction with micro-computed tomography images (Gadenne et al., 2019; Kollmann et al., 2011).

3.2 | Detection of *c002* or *shp* transcripts in the salivary glands of *A. pisum* head-thorax samples

We conducted two distinct experiments to detect individually by HRC RNA-FISH the expression of one of the two chosen target genes, either *c002* (ACYPI008617; XM_001948323) (Mutti et al., 2006, 2008), or *shp* (ACYPI009881; XM_001943863) (Rao et al., 2013). The residual autofluorescence of the cuticle and tissues that remains after treatment makes it easy to identify organs inside the heads, whatever the wavelengths of the lasers used (see images of the negative controls using Zeiss LSM 900 Confocal Microscope in Figure S2). *c002* gene transcripts were detected only in the salivary glands, in a subset of cells in the psg (Figure 2a), an observation consistent with the study by Mutti et al. (2008) that detected both mRNA and proteins of the *c002* gene in these glands. Similarly, the *shp* gene transcripts showed a restricted distribution in a subset of cells in the psg (Figure 2b), although their distribution is not identical to that of *c002*. These data match the SHP protein expression previously demonstrated on tissue sections of the salivary glands in *A. pisum* and *Metopolophium dirhodum* (Rao et al., 2013). Our results on a intact insect head confirmed the original observation for both chosen genes, thus validating our combined HCR RNA-FISH and iDisco+ clearing methods to detect transcripts without dissection of the target organs in aphids.

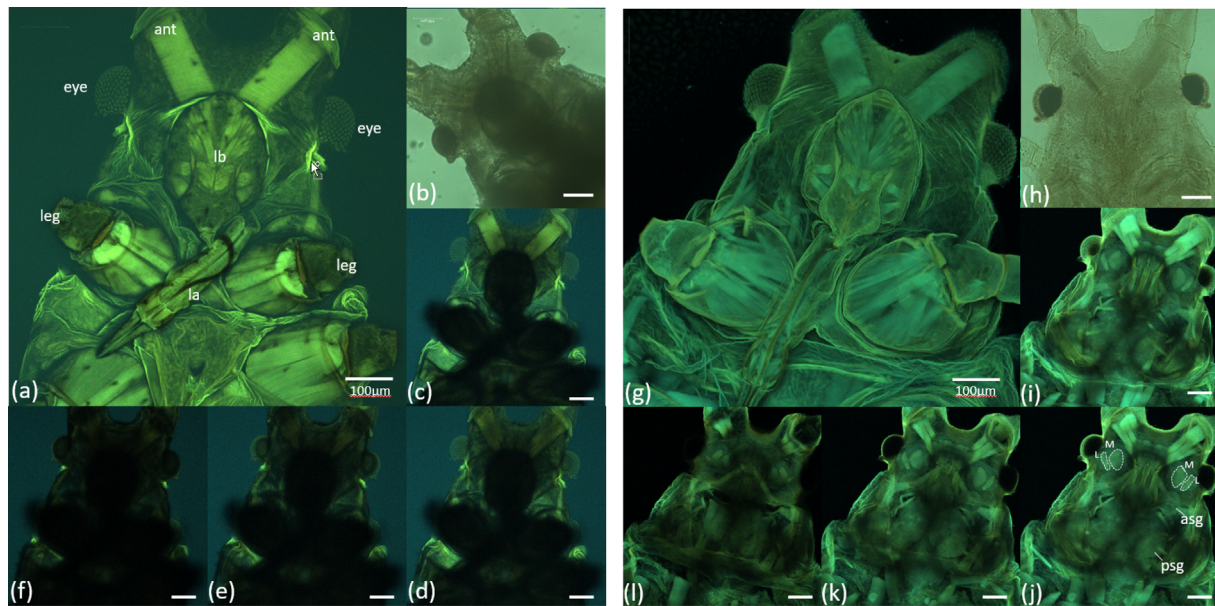


FIGURE 1 Comparison of widefield microscopic images of *Acyrthosiphon pisum* head-thorax samples optically cleared or not. (a–f) Widefield images of an untreated aphid head-thorax sample, ventral view. (g–l) Widefield images of an optical-treated aphid head-thorax sample. Note the differences observed in the levels of overall transparency of the specimens, with internal anatomical tissues only visible on the cleared sample (images [i–l] compared with images [c–f]). (a, g) Maximum intensity projections of confocal z stacks. (b, h) Differential interference contrast (DIC) images acquired with an extended depth of focus. (c–f; i–l) Partial maximum orthogonal projections from the ventral to the dorsal side of the same aphid individual, 20 μm of thickness each. The visual neuropils lamina and medulla are circled in (j). Scale bars: 100 μm . ant, antenna; L, lamina; la, labium; lb, labrum; M, medulla; psg, principal salivary glands.

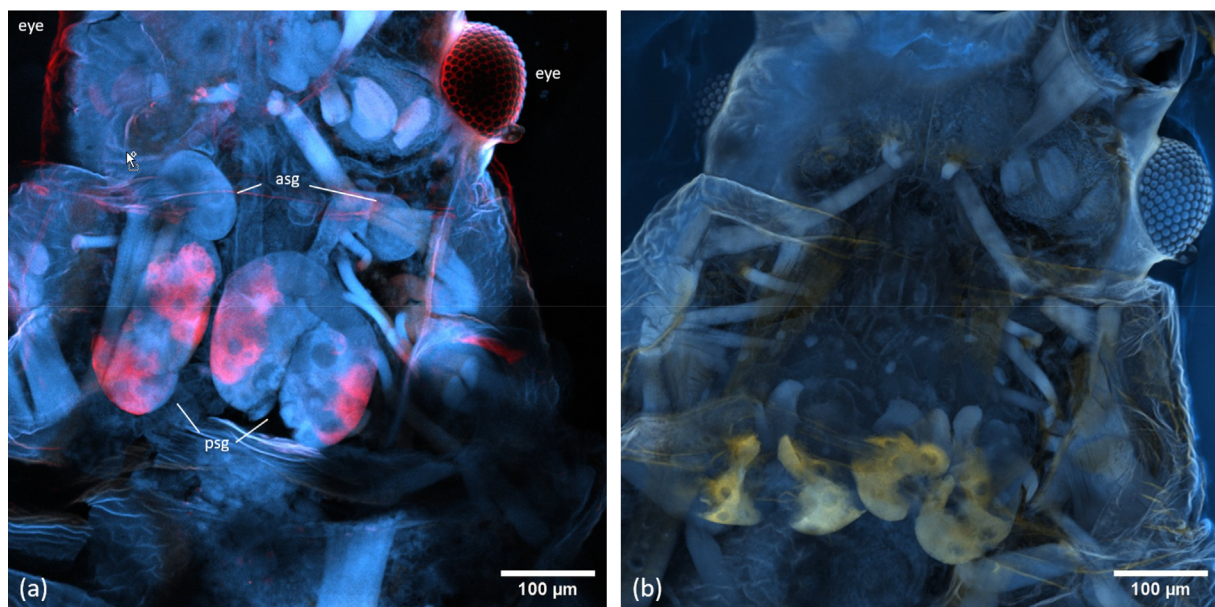


FIGURE 2 Detection of individual *c002* and *shp* transcripts using HCR-ISH in *Acyrthosiphon pisum* head-thorax samples. (a, b) Images are maximum intensity projections of confocal z stacks ventral view. (a) Detection of *c002* mRNA (Alexa 647, red). (b) Detection of *shp* mRNA (Alexa 546, yellow). Scale bars: 100 μm . asg, accessory salivary gland; psg, principal salivary gland.

3.3 | HCR RNA-FISH allows multiplex mapping of *c002* and *shp* transcripts in the salivary glands of *A. pisum* head-thorax samples

Due to the interest in characterizing the expression of multiple genes in a single sample, especially when studying spatiotemporal gene

expression, we applied our novel protocol to detect *c002* and *shp* gene transcripts simultaneously in the pea aphid salivary glands. We could highlight the two genes' expression in single head-thorax samples (Figure 3, a–g). Our images, and their analyses with DiANA (Gilles et al., 2017), clearly demonstrate that *c002* and *shp* transcripts do not colocalize in the same cells. Accessory and principal salivary glands

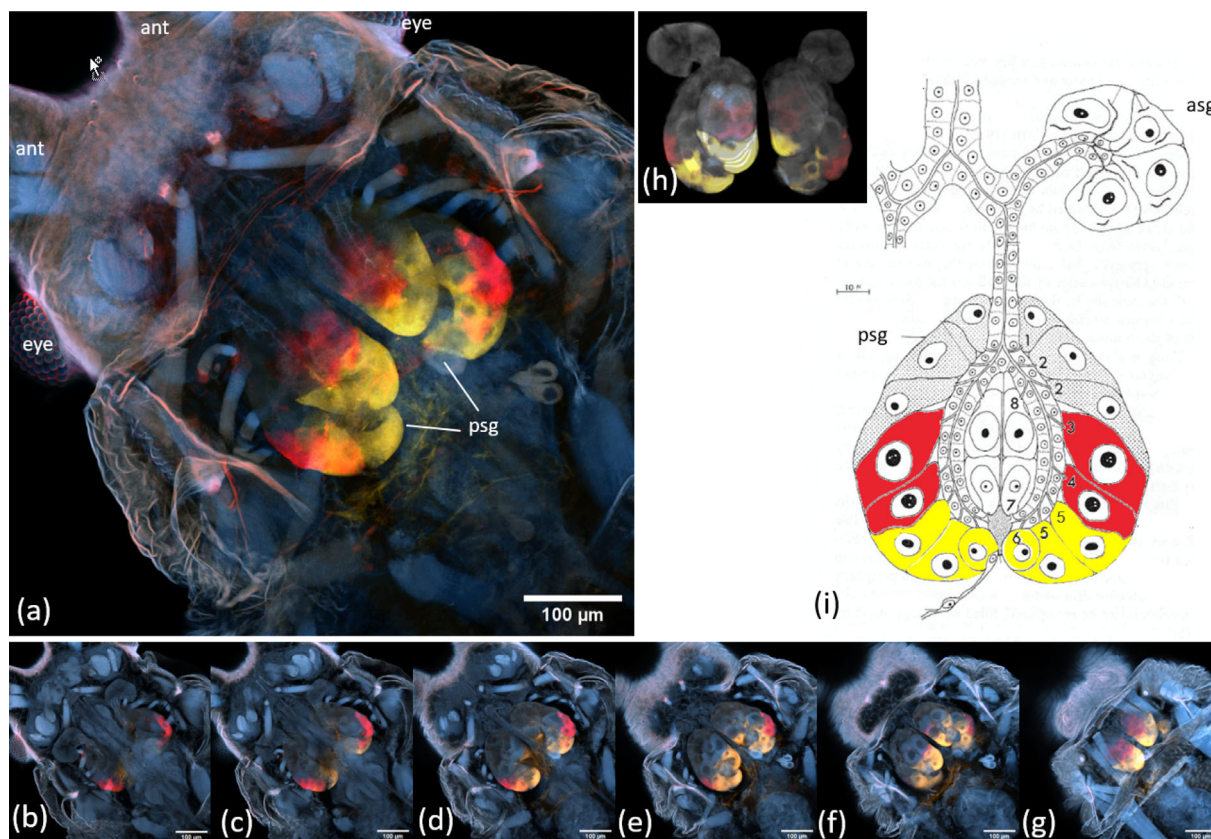


FIGURE 3 Multiplexed mapping of *c002* and *shp* transcripts in the salivary glands of a cleared aphid *Acyrthosiphon pisum* using HCR-ISH. (a) Image is a maximum intensity projection of confocal z stacks, ventral view. (b–g) Section planes from the ventral to the dorsal side of the same aphid individual, 9 μm of thickness each. (a–g) Detection of *c002* mRNA (Alexa 647, red) and *shp* mRNA (Alexa 546, yellow). (h) Screenshot of the salivary gland 3D reconstruction (see Movie S1) obtained with Image J's segmentation editor and 3D viewer plugins. (i) Schematic representation of a transverse section of one accessory salivary gland and one principal salivary gland showing cells expressing *c002* mRNA (red) and *shp* mRNA (yellow). Cell types are indicated (1–8). Within the principal salivary gland, type 1 and type 2 cells are electron-luscent, while types 3–8 are electron-dense. Adapted from Ponsen (1972). Scale bars: 100 μm . ant, antenna; asg, accessory salivary gland; psg, principal salivary gland.

are composed of 4 cells and 42 cells, respectively, each lobe of the psg consisting of 21 cells categorized according to their shape, cytoplasm type, nucleus, and nucleolus size (Ponsen, 1972). When comparing the drawing of salivary glands originally developed by Ponsen (1972) with our results, including a 3D reconstruction using segmentation (Figure 3h and Movie S1), we could precisely define the cells of the principal salivary glands expressing the two genes we analyzed (Figure 3i): cells types 3 and 4 for *c002* and types 5 and 6 for *shp*. As there are one cell type 3, two cells type 4, 6 cells type 5 and two cells type 6 per psg lobe (Ponsen, 1972), we can deduce that the expression of the effector *c002* is restricted to 16 cells in the pea aphid, while *shp* is expressed in 32 cells, all located in the principal salivary glands.

4 | DISCUSSION

The challenge when working on organisms not studied by a broad community is the availability of crucial and novel technology to advance research projects. Imaging technologies are paramount

to analyze in detail genes and protein expression in living organisms, and they allow outstanding progress in understanding complex biological processes and regulations. We implemented and validated for the first time a combined i-DISCO+ clearing and HCR RNA-FISH protocol for aphids on the *A. pisum* species. Our work allows the study of gene expression in 3D intact head samples in this non-model insect pest, providing a valuable research tool to the scientific community.

The importance of watery and gel saliva for aphid-plant interactions (van Bel & Will, 2016) prompted us to choose target genes expressed in the salivary glands. These organs in aphids' heads are close to other tissues (brain, eyes, muscles, epithelia, foregut, and midgut). This complex environment seemed an appropriate choice to develop our novel 3D protocol. To validate our approach, we selected two genes whose expression had been previously characterized in dissected salivary glands. The *c002* gene (ACYPI008617; XM_001948323) is essential for aphid feeding. Its expression in the pea aphid, both at the mRNA and protein level, was detected in a subset of cells that were not precisely assigned in dissected salivary glands (Mutti et al., 2008). The salivary sheath protein (ACYPI009881;

XM_001943863), initially identified in *A. pisum*' secreted saliva with a proteomic approach (Carolan et al., 2009), is a putative component of the salivary sheath. This protein expression was observed in dissected salivary glands of *A. pisum*, *M. dirhodum*, and *Sitobion avenae* species, detecting fluorescent signals in psg cells type 5 and type 6 and to a lesser extent in other cells (Rao et al., 2013).

We were able to visualize the cell-specific expression in salivary glands, thus validating the effectiveness of our protocol. Our combined approach enabled to identify very precisely the cells in which the target cells were expressed. For the first time, mRNAs of these two genes were detected in the same samples and with a 3D whole-body approach. We thus validated the multiple genes approach empowered by the HCR technology with the identification of two genes' mRNA expression contemporarily in intact aphid heads. The genes of interest expression analyses in 3D insects demonstrated that these two genes are expressed in distinct cells in aphids' salivary glands. These results suggest a certain level of cell specialization in this organ and underline the power of the 3D approach applied to intact insect parts.

In conclusion, we validated an accurate 3D imaging approach to detect aphid molecules in their anatomical context without the need to dissect tissues. The protocol presented here opens the possibility of studying gene expression in aphid samples without disrupting tissue organization, and this is a need when studying tiny-sized organs that are difficult to analyze using a section 2D approach.

AUTHOR CONTRIBUTIONS

Bastien Cayrol: Conceptualization; investigation; methodology; formal analysis; writing – original draft. **Stefano Colella:** Conceptualization; formal analysis; writing – original draft; writing – review and editing; supervision. **Marilyne Uzest:** Conceptualization; writing – original draft; writing – review and editing; formal analysis; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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