DNASCRIPT

APPLICATION NOTE

Single Molecule RNA FISH (smFISH)

***In situ* hybridization methods, such as single molecule RNA FISH (smFISH) used in spatial transcriptomics, rely on the availability of fluorescent probes. In this Application Note, we present the first report of viral smFISH in the gut of *Drosophila melanogaster*, performed with fluorophore-labeled DNA probes produced in-house with DNA Script's Enzymatic DNA Synthesis (EDS) technology and benchtop SYNTAX System. EDS-powered smFISH enables rapid and cost-effective probe production for smFISH assay development and optimization; thereby providing a fast, robust, and highly customizable alternative to immunofluorescence.**

INTRODUCTION

Spatial transcriptomics is a rapidly growing, transformative field of biology. Enabled by technological advances in biological analysis, it allows for gene expression profiling in biologically relevant spatial contexts. Fluorescence i*n situ* hybridization (FISH)[1] is an established technology that employs labeled probes to interrogate nucleic acids in tissue samples, and forms the basis for several spatial transcriptomics methods (see Asp et al., 2020[2] for a recent review). Of these, single molecule RNA FISH (smFISH)[3,4] and variations thereof[5] offer a quick and simple strategy for the detection of single RNA molecules in fixed cells.

Hybridization probes for smFISH may be designed and sourced within a matter of days, as opposed to the months needed to produce the reagents for traditional, antibody-mediated immunofluorescence methods. Nevertheless, smFISH assays are often considered to be cost prohibitive and time consuming due to the need for dozens of custom, modified (fluorophore-labeled) oligonucleotides.

DNA Script's proprietary Enzymatic DNA Synthesis (EDS) technology and SYNTAX benchtop DNA printer[6,7] enable same-day synthesis of DNA oligos labeled with a range of different fluorophores in any molecular biology lab, at competitive costs compared to probes sourced from leading oligo suppliers. This supports rapid iteration of probe design and fine-tuning of assay specificity and sensitivity, thereby accelerating the design-test-build-learn cycle.

This Application Note provides proof-of-concept for the use of enzymatically synthesized probes in smFISH, in a seminal study of natural viral infection of *Drosophila melanogaster*.

D. melanogaster (Figure 1) is a canonical model organism utilized across a range of biological disciplines, from genetics to immunology and the basis of antimicrobial resistance. It has proven particularly valuable in advancing our understanding of virus-host interactions and how the co-evolutionary dynamics between virus and host shape immune system function.[8] These studies are particularly relevant to the natural transmission of human and agricultural pathogens with insect hosts.

Wild populations of *D. melanogaster*, as well as many laboratory stocks, are often persistently infected with a variety of naturally occurring viruses.[9,10] Two of the most common examples are Drosophila C virus (DCV) and Drosophila A virus (DAV); RNA viruses that can be found in

FIGURE 1. THE LESSER FRUIT FLY, *DROSOPHILA MELANOGASTER*, a model organism used since the early 1900s across many areas of biological research due to its relatively simple genetics and rapid life cycle.

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approximately 40% of wild populations.[8,11,12] While DCV is the most studied natural pathogen of *D. melanogaster*, little is known about natural DAV infection.

The Saleh laboratory (Institut Pasteur, Paris, France) recently reported that DAV actively replicates in *D. melanogaster* upon oral infection, evidenced by the detection of negative-strand viral RNA by RT-qPCR.[9] Preliminary immunofluorescence studies suggested that DAV naturally infects the *D. melanogaster* gut (unpublished results).

To confirm these results, oligos targeting the DAV genome were designed. Fluorophore-labeled smFISH probes were produced in-house using the SYNTAX System. Using these probes, DAV infection in *D. melanogaster* guts was demonstrated for the first time. Implementation of EDS technology in smFISH workflows extends the utility of this technology as a fast and simple alternative to immunofluorescence .

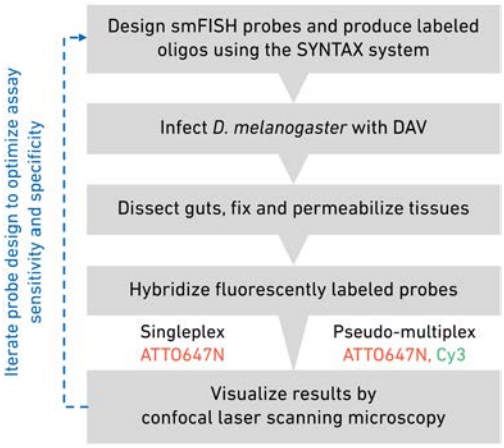

FIGURE 2. OVERVIEW OF THE smFISH WORKFLOW USED IN THIS STUDY. DAV probes were designed and synthesized, flies were infected with DAV, and guts were processed as described in *Materials and Methods*. Results presented here were generated with two sets of 24 optimized DAV-specific probes, derived from several cycles of quick iteration enabled by on-demand, in-house probe synthesis with the SYNTAX System.

EXPERIMENTAL DESIGN

Single molecule RNA FISH (smFISH) was performed as outlined in Figure 2 and described in *Materials and Methods* (p. 6). In short:

- *Drosophila melanogaster* flies were infected with Drosophila A virus (DAV) as described in Nigg et al, 2021.[9]
- Following gut dissection, tissue samples were fixed and permeabilized. Hybridization was performed with smFISH probes targeting the DAV genome. A pool of 24 probes (3'-labeled with the red-emitting fluorophore, ATTO647N) was utilized in a singleplex approach, whereas two pools of 24 probes each (targeting different regions of the DAV genome and 3'-labeled with ATTO647N or green-emitting Cy3, respectively) were employed in a pseudo-multiplex protocol. Results were visualized by confocal microscopy.

RESULTS AND DISCUSSION

Results obtained with the DAV-specific, ATTO647N-labeled probe set in a singleplex smFISH assay suggest successful infection of *Drosophila melanogaster* gut cells after oral infection with DAV (Figure 3A, four representative **DAV+ Probe+** images). Uninfected (**DAV- Probe+**) and infected no-probe (**DAV+ Probe-**) controls showed no red fluorescence. All tissues were stained with the blue-emitting nuclear stain 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. The presence of DAV in gut cells was confirmed by indirect immunofluorescence, performed with a primary antibody targeting the DAV capsid protein and a fluorophore-labeled (red-emitting) secondary antibody (Figure 3B).

Representative results from the repeat singleplex smFISH experiment, performed with a replicate set of ATTO647N-labeled probes, are given in Figure 4. The presence of DAV RNA was again clearly demonstrated. In both experiments, variation in the intensity of the red signal suggest differences in viral genome copy number within individual cells, with brighter fluorescence corresponding to more DAV genome copies. This may be attributed to variation in the viral infection cycle stage or differences in viral infection load within specific cell types.

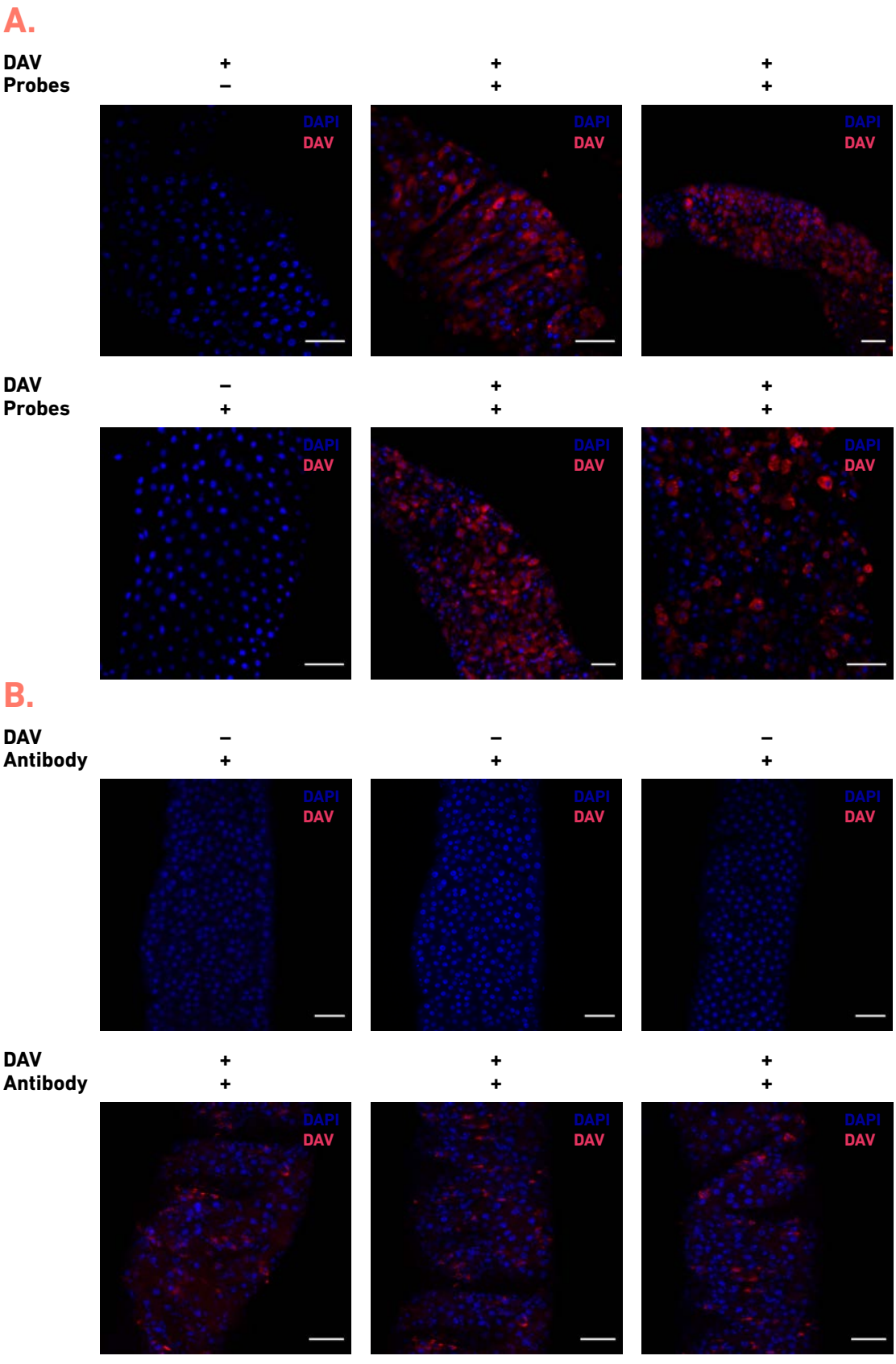

FIGURE 3. FLUORESCENT IMAGING OF DAV-INFECTED *D. MELANOGASTER* GUT CELLS. A. Singleplex smFISH assay performed with DAV-specific, red-emitting probes synthesized by EDS. **B.** Immunofluorescent indirect staining of a DAV-infected tissue with a viral capsid-specific primary antibody and a red-emitting secondary antibody. Size bar: 50 µm.

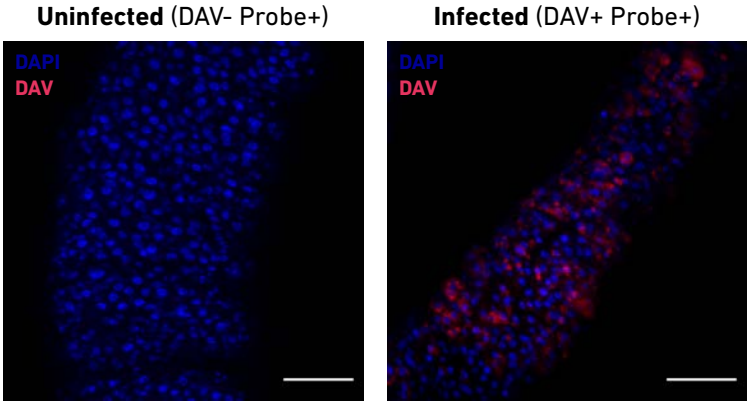

FIGURE 4. REPEAT SINGLEPLEX SMFISH ASSAY TARGETING THE DAV GENOME, performed with the same set of ATTO647N-labeled DAV-specific probes, but from an independent SYNTAX synthesis run. Blue fluorescence corresponds to cell nuclei stained with DAPI. Size bar: 50 µm.

A third experiment was performed to assess the performance of enzymatically synthesized probes in a "pseudo-multiplex" smFISH assay (Figure 5). Gut tissue was stained with the same ATTO647N-labeled probe set used previously and with a set of 24 Cy3-labeled probes targeting different regions of the DAV genome. Both probe sets identified DAV RNA in the same cells, suggesting potential DAV infection and replication. Further experimentation aims to determine the specific cell types within the *D. melanogaster* gut that are susceptible to DAV infection.

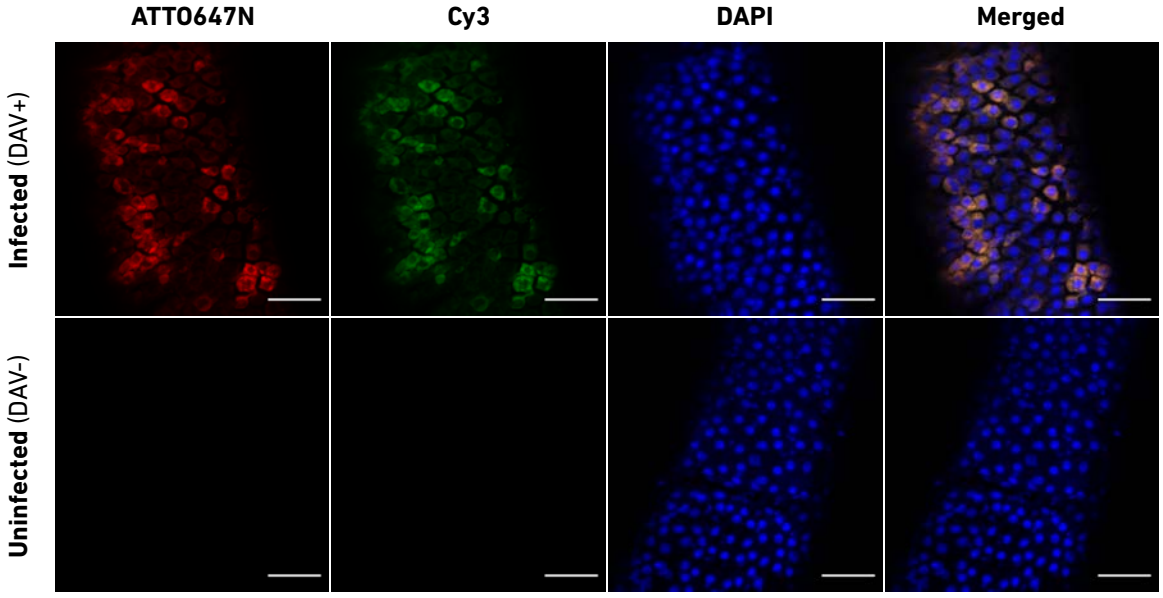

FIGURE 5. PSEUDO-MULTIPLEX SMFISH ASSAY PERFORMED WITH DIFFERENTIALLY LABELED DAV-SPECIFIC PROBES. All tissues, including uninfected (DAV-) controls in the bottom row, were stained with DAPI and both DAV-specific probe sets. Images were captured in individual channels: ATTO647N (red), Cy3 (green), or DAPI (blue) as indicated and were merged in the last column. Size bar: 50 µm.

CONCLUSION

The data presented here represents the first demonstration of single molecule viral RNA FISH in *Drosophila melanogaster* gut cells. Drosophila A virus (DAV) RNA was successfully detected in putatively infected cell populations using two sets of 3'-labeled probes (with different fluorophores) produced with DNA Script's EDS technology. The presence of viral capsid in these cells was confirmed by immunofluorescence. Future work will focus on determining whether the cellular tropism and/or the spatial distribution of DAV RNA within the gut changes over the course of infection.

Although a side-by-side comparison with smFISH probes synthesized using conventional phosphoramidite chemistry was not included in this study, EDS probes were found to meet the specificity and sensitivity requirements for smFISH assays, and perform equivalently to smFISH probe sets sourced for other studies from a leading oligo supplier. EDS probes were also demonstrated to support both single- and pseudo-multiplex viral smFISH.

Single molecule RNA FISH offers several advantages over traditional immunofluorescence for *in situ* detection of viruses (Table 1). These include:

- reproducibility (unlike DNA-based probes, custom antibody preparations can never be replaced with identical reagents),
- tunability (unlike antibody-based reagents, probe sensitivity and specificity can be easily optimized through oligo redesign and the addition/subtraction of different oligos from a pool), and
- scalability (the multiplexing capacity of immunofluorescence is constrained by the species of origin of primary and secondary antibodies, while smFISH assays are, in principle, only limited by the constraints of the microscope).

Another significant advantage of smFISH over immunofluorescence lies in the time required to obtain custom reagents. Turnaround times for antibody-based reagents range from weeks to months, whereas fluorophore-labeled DNA oligos are typically available within a matter of days. In-house oligo production with DNA Script's EDS technology and benchtop DNA printer further extends this advantage through same-day availability of smFISH probes. This reduces the overall turnaround time from probe design to result to <3 days—less than half the time needed when probes are ordered from an external supplier, and supports rapid iteration of probe panels and overall assay optimization.

TABLE 1. COMPARISON OF FLUORESCENT DETECTION METHODS AND PROBE SYNTHESIS CHEMISTRIES.

Parameter	Immunofluorescence	smFISH (chemically synthesized probes)	smFISH (EDS probes)
Target	Viral protein	Viral RNA	
Protocol length	Overnight + 3 – 4 hours	Overnight	
Probe generation time	≥45 days	≥5 days	Overnight
Probe reproducibility	Variable	Strong	
Probe fine-tuning	Not possible	Cost prohibitive	Cost effective
Multiplexing capacity	Limited	Broad	

MATERIALS AND METHODS

Probe design and production

Single molecule RNA FISH (smFISH) probes complementary to the positive strand of the DAV genome (two sets of 24 probes each, targeting different genomic regions) were designed using the Oligostan software.[5] Oligos ranged between 28 and 32 bp in length, and were produced using the SYNTAX System (DNA Script part number 100296 or 100094). The first probe set was synthesized with an ATTO647N fluorophore at the 3'-end of each oligo, whereas oligos in the second set were 3'-labeled with Cy3. The first probe set was synthesized in duplicate during two independent runs. Probe pools for hybridization experiments were created by mixing equal volumes of ready-to-use, desalted, quantified and normalized oligos produced with the SYNTAX System.

Infection of Drosophila with DAV

Infection of *Drosophila melanogaster* with Drosophila A virus (DAV) was performed as described in Nigg et al., 2021.[9] Briefly, mated adult female w1118 flies were starved for 5 hours, after which oral inoculation was performed by means of a cornmeal diet containing 100 µL of undiluted DAV stock. Flies were separated into groups of 40 and allowed to feed on the DAV-coated cornmeal for 24 h at 25°C. Fresh food was subsequently provided at two-day intervals, until experiments were performed.

Single molecule RNA FISH (smFISH) in *Drosophila* guts

Drosophila guts were dissected in phosphate-buffered saline (PBS) over the course of 20 min, and incubated in fixative (4% paraformaldehyde, 0.3% Tween-20) for 20 min with gentle rotation. Guts were rinsed (2 x 2 min) in PBT (1X PBS + 0.1% Triton-X 100), incubated in permeabilization buffer (1X PBS + 0.5% Triton-X 100) for 20 min, and washed in fresh wash buffer (10% deionized formamide in 2X SSC). Guts were subsequently incubated in 250 µL of prewarmed hybridization buffer (10% deionized formamide, 5% dextran sulphate in 2x SSC) containing the probe pool at a final concentration of 200 nM. Incubations were performed for 16 h in a dark, humidified chamber (37°C) with rotation (300 rpm). On the following day, gut tissues were consecutively rinsed with fresh wash buffer, SSC, and PBT containing 1 µg/mL of DAPI. Stained tissue samples were mounted in N-propyl gallate mounting medium (1.25% n-propyl gallate, 75% glycerol) and visualized using confocal laser scanning microscopy.

Immunofluorescent staining of DAV-infected Drosophila gut cells

Drosophila guts were dissected in PBS as described previously, followed by incubation in 4% paraformaldehyde for 2 h with gentle rotation. After rinsing with PBT, tissues were incubated in 50% glycerol for 30 min, in PBT for 10 min, and overnight at 4°C with the primary antibody. On the following day, gut tissues were washed with PBT. Tissues were subsequently incubated in appropriately diluted secondary antibody, for 3 h at room temperature with gentle rotation. Three additional washes with PBT were performed, with the final wash containing DAPI (1 µg/mL). After a final incubation in 50% glycerol for 30 min, stained gut tissue was mounted in N-propyl gallate mounting medium (1.25% n-propyl gallate, 75% glycerol) and visualized using confocal microscopy.

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