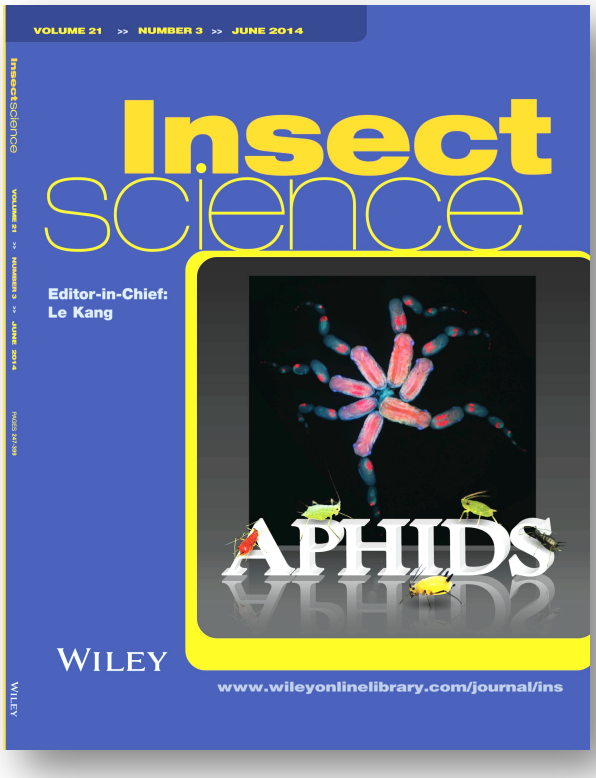
Reliable protocols for whole-mount fluorescent *in situ* hybridization (FISH) in the pea aphid *Acyrthosiphon pisum*: A comprehensive survey and analysis

Chen-yo Chung[1], Charles E. Cook[2], Gee-way Lin[1,3], Ting-Yu Huang[1,4] and Chun-che Chang (張俊哲)[1,3,5]

[1]*Laboratory for Genetics and Development, Department of Entomology/Institute of Biotechnology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan,* [2]*European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Trust Genome Campus, Hinxton, Cambridge, UK,* [3]***Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan,*** [4]*Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan, and* [5]*Genome and Systems Biology Degree Program, National Taiwan University, Taipei, Taiwan*



Abstract RNA *in situ* hybridization (ISH), including chromogenic ISH (CISH) and fluorescent ISH (FISH), has become a powerful tool for revealing the spatial distribution of gene transcripts in model organisms. Previously, we developed a robust protocol for whole-mount RNA CISH in the pea aphid *Acyrthosiphon pisum*, an emerging insect genomic model. In order to improve the resolving capacity of gene detection, we comprehensively surveyed current protocols of whole-mount RNA-FISH and developed protocols that allow, using confocal microscopy, clearer visualization of target messenger RNAs (mRNAs) – including those subcellularly localized and those with spatially overlapping expression. We find that Fast dye-based substrate fluorescence (SF), tyramide signal amplification (TSA), and TSA Plus all enable identifying gene expression thanks to multiplex amplification of fluorescent signals. By contrast, methods of direct fluorescence (DF) do not allow visualizing signals. Detection of a single gene target was achieved with SF and TSA Plus for most mRNAs, whereas TSA only allowed visualization of abundant transcripts such as *Apvas1* and *Appiwi2* in the germ cells. For detection of multiple gene targets using double FISH, we recommend: (i) TSA/TSA, rather than TSA Plus/TSA Plus for colocalized mRNAs abundantly expressed in germ cells, as proteinase K treatment can be omitted; and (ii) SF/TSA Plus for other gene targets such as *Apen1* and *Apen2* as inactivation of enzyme conjugates is not required.

SF/SF is not ideal for double FISH experiments due to signal blurring. Based on these new conditions for RNA-FISH, we have obtained a better understanding of germline specification and embryonic segmentation in the pea aphid. We anticipate that the RNA-FISH protocols for the pea aphid may also be used for other aphids and possibly other insect species, thus expanding the range of species from which useful insights into development and evolution may be obtained.

Key words aphid, fluorescence, gene expression, *in situ* hybridization, model organism, probe detection

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