Project Report

Abstract:
Important insights into gene regulation can be gained through the study of cell-to-cell variation and subcellular localization of RNA. Plant biologists lacked the tools to explore these aspects of regulation until development of a single molecule Fluorescent In situ hybridization (smFISH) method for Arabidopsis root meristem cells (Duncan et al., 2016). OpenPlant funding enabled us to promote this method for RNA imaging and quantification widely across the plant science community. It also enabled us to clarify imaging limitations before embarking on the first co-ordinated attempt to extend this technology into a wider range of plant tissues and species. Our work has provided a strong foundation for improved RNA imaging that will open up numerous avenues for Arabidopsis and Marchantia research in the future.

Report Body

Below is a summary of the progress made toward our three project objectives:

1) Promoting the existing smFISH method more widely across the plant science community:

- Posters describing smFISH work were presented at the 2016 RNA Society conference in Kyoto and OpenPlant Forum in Norwich.
- An editor from the journal Transcription invited submission of a commentary article after seeing the poster in Kyoto. (This manuscript is due for submission in May).
- Whilst in Japan, Susan gave invited smFISH seminars to plant scientists at Kobe University, the University of Tokyo and RIKEN Institute in Yokohama.
- Following invitations, details of the existing method have now been submitted to the open platform Bio-Protocol (http://www.bio-protocol.org) and the smFISH probe manufacturers, LGC (http://www.biosearchtech.com). Once published online, these versions of the protocol will increase open access options for researchers wishing to find out more about this technology.
- In addition to extending the existing smFISH method to the Haseloff lab in Cambridge, ongoing support has been provided to other groups interested in this method – most notably to labs led by Prof. Anne Osbourn (JIC) and Prof. Fujiwara (University of Tokyo).
- In recognition of her ongoing efforts to promote plant smFISH, LGC has made Susan an expert collaborator. The company will present posters at both the Plant and Animal Genome Asia and Asia Plant Genomics and Gene Expression conferences to explicitly promote Susan's method.
- Susan discussed the advantatges and disadvantages of single molecule RNA FISH during an interview with a free lance journalist writing a piece for the BioCompare website (http://www.biocompare.com).
- There are ongoing discussions for Susan to organise an LGC funded workshop that would enable researchers to visit Norwich and gain hands-on experience of smFISH.
- Susan has been invited to present her smFISH work at the Norwich Single Cell Symposium at the Earlham Instute in May.

2) Clarify minimal microscopy equipment set up required for smFISH imaging.

- The minimum requirements for smFISH imaging have been clarified together with Dr. Stefanie Rosa (University of Potsdam): A wide-field fluorescence microscope, 63x or 100x oil immersion objective (>1.3 NA), mercury or metal halide light source, suitable filter sets, a EM-CCD camera is preferable, but a CCD camera optimised for low light imaging rather than speed have been found to provide good images. These details have been added to the two most recent open versions of the protocol.

3. Adapting smFISH for use in other Arabidopsis tissues and Marchantia gemmae:

Information coming from discussions with RNA experts and cell biologists working in a range of disciplines was taken into consideration when devising two strategies for advancing RNA imaging in plants. A summary of our progress is outlined below:

- *Approach 1: DIY Probes*

A logical approach was taken to design probes capable of amplifying individual RNA signals. We adapted a method previously published by Sinnamon and Czaplinski (*Methods Mol. Biol.* 2015) where a series of hybridization steps are used to create branched DNA structures that can be decorated with multiple fluorescent dye molecules.

Four main conclusions came from a series of experiments using the DIY probes:
1) Enabled mRNA detection on both widefield and confocal systems
2) Provided up to 5.8x signal amplification
3) Exhibited improved fluorophore photostability
4) Were markedly less sensitive and specific than standard smFISH probes

- *Approach 2: Affymetrix Probes*

Affymetrix probes also amplify RNA signals via a branched DNA approach, but they employ patented sequences that are specifically designed to minimize off-target binding. Initial tests for these probes were very encouraging. Although there was insufficient time to exhaustively assess probe performance, subcellular distribution and quantifications suggested acceptable levels of RNA sensitivity and specificity for these commercial probes. The Affymetrix system offered three clear advantages over the existing smFISH method:

1) An option to image three, rather than two, RNA targets simultaneously
2) Amplified signals were compatible with confocal imaging
3) RNA could be visualized in more differentiated root cells, e.g. root hairs.

After successfully using Affymetrix probes to label RNA in Arabidopsis root cells, we decided to adopt and amend this technology for RNA imaging in *Marchantia polymorpha* gemmae. Firstly, we determined optimal tissue handling and clearing techniques and then devised an *in situ* protocol that successfully produced confocal images of RNA in the gemmae. Further experiments are now required to validate and rigorously test the specificity of these probe signals.

Budget

Reagents for Approach 1: DIY Probes	1564.66
Reagents for Approach 2: Affymetrix Probes	2309.24
Conference Posters	60.00
TOTAL	**3933.90**

Follow on plans

We request ongoing funds to purchase additional sets of smFISH Quasar670® Stellaris probes and Alexa488® Affymetrix probes that are complimentary to sequences shared between Venus and GFP RNA transcripts. These would enable us to validate our signals, optimize protocol steps and determine the limitations of RNA imaging in a wide range of Arabidopsis and Marchantia lines. Remaining funds will be used to cover train travel between Norwich and Cambridge and accommodation costs. We anticipate that these experiments will be completed over the next six months.

Alexa488® Affymetrix probe set:	£261.00
Quasar670® LGC probe set:	£379.00
Travel and accommodation:	£360.00
TOTAL:	**£1000.00**