How to use GFP-tagged proteins in cell biology

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Background

Freitag *et al*. (2004) have reported successful expression of several GFP-tagged genes, using the *N. crassa ccg-1* promotor, and inserted at the *his-3* locus on linkage group I. They used GFP-tagged *histone H1* and *Bml* (*β-tubulin*) genes for analyzing the nuclear and microtubule dynamics in growing vegetative hyphae and in developing asci and ascospores. When homozygous, the ectopically inserted *hH1::GFP* is expressed throughout meiosis and ascospore development. However, when it is heterozygous, the ectopic as well as the native copies of the gene are silenced during meiosis and postmeiotic mitosis. The silencing does not extend into the ascospore maturation stage, and *hH1::GFP* nuclei in four of the eight ascospores begin to fluoresce ~18-24 hours after delimitation (Raju *et al.* unpublished). Shiu *et al*. (2001) discovered the meiotic silencing phenomenon without the benefit of GFP-tags. The GFP-tagged genes now provide a powerful and more direct visual demonstration of the localization of the fusion protein and the temporal limits of meiotic silencing during ascus development (Figure 1).

Procedure

Crosses for cytology. Crosses for cytology are routinely made in Petri plates on synthetic crossing medium supplemented with 1% sucrose and 2% agar. The protoperithecial parent is first grown for 5 days at 25^{o}C and then fertilized by adding conidia from the second parent. After fertilization, perithecia begin to develop more or less simultaneously although the asci within a perithecium develop asynchronously. The oldest asci in each perithecium are at about the same stage of development. Each perthecium produces well over 200 asci and the oldest asci eject mature ascospores about 8-10 days after fertilization at 25^{o}C. The developing perithecia (unfixed, unstained) are dissected at 12-24 hour intervals from 3 to 10 days (after fertilization), in a drop of 10% glycerol (90% water) on a glass slide, lightly squashed under a cover glass. Edges or the glass are sealed with melted dental wax or nail polish. Glycerol helps reduce surface tension, thus avoiding air bubbles under the cover glass, and it also prevents the slides from drying quickly.

Fluorescence microscopy. The construction of gene fusions, transformation protocols, and the potential uses of the GFP tags are given in Freitag *et al*. (2004). Raju's description here provides advice on the set up and use of a fluorescence microscope for examining the expression and localization of GFP-tagged histone H1 in the developing asci and ascospores. Any research microscope (*e.g.* Zeiss, Leitz, Nikon, or Olympus) equipped with epifluorescence illumination can be tailored for GFP observations. Our Nikon Microphot FX microscope is fitted with a filter set, specifically suited for a variant of GFP (sGFP), from Chroma (#41012: 480/40 nm excitation filter, 505 nm dichroic mirror, 510 nm long pass emission filter). We use Zeiss Neofluar 10× (NA 0.30) Nikon Plan 20×(NA 0.50), and Nikon Fluar 40× Oil (NA 1.30) objectives for routine observations at 100× to 500× magnification. The 10x objective is well suited for observing and imaging large rosettes of maturing asci, the 20x for imaging 5-10 asci and the 40×for imaging 1

to 5 asci at high magnification. A low intensity back lighting through a dark-field condenser provides the necessary contrast, and ascus and ascospore outlines.

Digital imaging. For digital imaging, a Nikon CoolPix 5000 is attached to the video port of Nikon Microphot FX with three adapters: A Nikon C-mount adapter on the microscope's video port, a photo eyepiece-like lens adapter (MM-Cool from Microscope World), which connects to the C-mount adapter at one end, and to a Nikon camera adapter tube (UR-E6) at the other end. The UR-E6 in turn screws onto the outer mount of the camera lens. I have recently adapted another camera (CoolPix 5400) to the Nikon microscope with MM3-45 (MM-3XS + M4537) from Microscope World. Additional advice and appropriate adapters for several Nikon, Sony, Canon, and Olympus digital cameras may be obtained from the camera manufacturers/distributors or from Microscope World (www.microscopeworld.com). Microscopes without the C-mount adapter may also be used by connecting the camera and the MM-Cool (or a similar adapter) directly to the phototube or to one of the binocular eyepiece ports with the supplied 23 mm sleeve.

The images of fluorescing nuclei in the developing asci and ascospores are fairly bright when observed through the microscope (Figure 1). However, the images are dim and hardly visible on the built-in small, camera screen. Thus, it is necessary to frame and focus images of asci in bright field, and then switch the filter set and light path to GFP imaging. Another trick is to increase the ISO setting to 800, when the GFP images appear brighter for focusing, then change the ISO setting back to 100 for exposures. At ISO 100, the 5-megapixel sensors in both CoolPix cameras are capable of recording high definition images. For fluorescence imaging, typical exposure times are set manually at 4-8 sec (F 5.4) with 10× objective, 2-4 sec with 20× and 1-2 sec with 40×. For bright field imaging the camera is set in Program mode; the built-in flash is, of course, disabled for both bright field and fluorescence imaging. Generally, I find the images on CoolPix 5400 screen are brighter than on CoolPix 5000 screen, perhaps because of different sensors or lens adapters on the two cameras.

For use of Red Fluorescent Protein, see Freitag and Selker (2005).

References

Freitag, M., and E. U. Selker. 2005. Expression and visualization of Red Fluorescent Protein (RFP) in Neurospora crassa. Fungal Genet. Newslett. 52: 14-17.

Freitag, M., P. C. Hickey, N. B. Raju, E. Selker, and N. D. Read. 2004. GFP as a tool to analyze the organization, dynamics, and function of nuclei and microtubules in *Neurospora crassa*. Fungal Genet. Biol. 41: 897-910.

Shiu, P.K.T., N. B. Raju, D. Zickler, and R. L. Metzenberg. 2001. Meiotic silencing by unpaired DNA. Cell 107: 905-916.

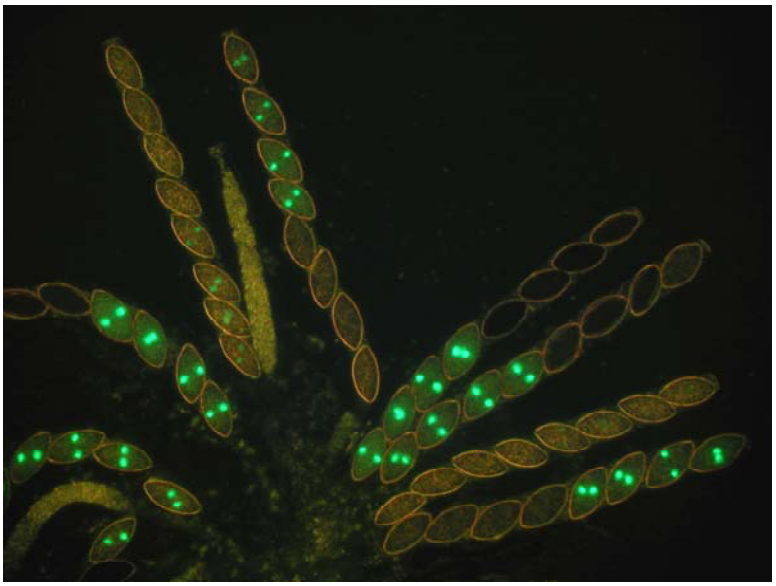

Fig 1. *N. crassa*. Maturing asci from Wild type x histone H1-GFP. Histone H1 being a chromosomal protein, the GFP-tagged nuclei (two per spore at this stage) glow in four of the eight ascospores; the remaining four ascospores carry the untagged nuclei from the wild-type parent. Histone H1 was completely silenced in the developing asci until ascospore delimitation, but it is expressed in the developing ascospores. The nuclear glow is brighter in older asci. Photo credit: N.B. Raju.