A bioluminescent *Agrobacterium tumefaciens* for imaging bacterial activity *in planta*

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Abstract

Bioluminescence enables the monitoring of spatio-temporal dynamics and activity of bacterial populations *in planta*. We here describe a procedure to use AgroLux, a bioluminescent *Agrobacterium tumefaciens*, as a tool to study bacterial responses upon agroinfiltration. The first method details how to transform bioluminescent AgroLux to carry binary plasmids of interests. Then, a simple agroinfiltration assay for *in planta* imaging of bioluminescence signals is presented. AgroLux assays will increase our understanding of plant-Agrobacterium interactions and plant immunity and improve molecular farming.

Keywords: *Agrobacterium tumefaciens*, agroinfiltration, AgroLux, bacterial bioluminescence, *Nicotiana benthamiana*

1 Introduction

Bioluminescence has reshaped imaging techniques for living organisms [1]. *In vivo* bioluminescence in bacteria can be obtained upon the expression of two luciferases (luxA and luxB) and three enzymes (luxC, luxD, luxE) that synthesise the long-chain aldehyde substrate [2]. These enzymes are encoded by the *lux* operon, which can be transferred and integrated into a bacterial genome to enable autonomous photon emission. Photons can be captured by charge-coupled device (CCD) cameras to study spatio-temporal dynamics of bioluminescence in bacterial populations [3-4].

Bioluminescence-based imaging is increasingly used in plant-bacteria interactions to monitor bacterial localisation and activity *in planta* [5]. It allows the detection of living bacteria as light emission depends on ATP availability and therefore active metabolism [6]. Pathogenic bacteria tagged with the *lux* operon are also used to investigate disease resistance and plant immune responses on bacterial fitness [7-8].

We recently demonstrated that bioluminescence could be used accurately and reliably to report *Agrobacterium tumefaciens* activity in agroinfiltrated leaves [9]. Agrobacterium-mediated transient expression (agroinfiltration) is frequently used to deliver genes of interest into a host plant [10]. However, little is known about Agrobacterium in agroinfiltrated tissues and how the bacteria respond to host plant immune defences. Therefore, we have generated AgroLux to study Agrobacterium viability *in planta* post-agroinfiltration [9]. AgroLux is derived from *A. tumefaciens* strain GV3101 and contains the *lux* operon integrated into its genome. AgroLux bioluminescence signals have been used to improve transient expression efficiency and to study plant immunity [9]. The broad versatility of AgroLux and its capacity to track bacterial activity *in planta* can be used to explore new biological questions.

Here we describe the detailed procedure to use AgroLux to monitor Agrobacterium activity *in planta* upon agroinfiltration. We first detail a method to prepare AgroLux competent cells and transform AgroLux with binary plasmids of interest, as bioluminescence is not impairing Agrobacterium-mediated transient protein expression [9]. We then use the widely adopted expression host *Nicotiana benthamiana* as an example to monitor and quantify Agrobacterium bioluminescence signals. AgroLux provides a valuable non-destructive and easy-to-use imaging tool allowing insight into Agrobacterium responses in agroinfiltrated plant tissues. For example, this tool could be used to optimise the infiltration process in order to boost recombinant protein levels in agoinfiltrated leaves.

2 Materials

2.1 Plants

1. *N. benthamiana* plants are used in this article as an example (*see* **Note 1**). Plants are sown in M2 compost and grown for five weeks in a greenhouse at 22 °C under a 16/8 h light/dark regime.

2.2 AgroLux

1. Bioluminescent *Agrobacterium tumefaciens* strain AgroLux (*see* **Note 2**) is used for leaf agroinfiltration. This AgroLux strain can be transformed to carry binary plasmids for transient recombinant protein expression (*see* **Note 3**).

2.3 Laboratory tools and materials

1. Image Quant LAS4000 imaging system (GE Life Sciences), or any chemiluminescence imaging system.
2. Infinite M200 plate reader (Tecan Group Ltd).
3. Plant growth cabinet (E-59, Percival Scientific or similar).
4. Greenhouse.
5. Centrifuge (for 50 mL falcon tube).
6. Standard image scanner (Epson Scanner or similar).
7. Spectrophotometer (Jenway 7200 or similar).
8. Shaking incubator (180 rpm at 28 ^{o}C).
9. Cork borer (6 mm diameter).
10. White or black 96-well plate.
11. Needle-less 1 mL syringes.

2.4 Buffers and other solutions

1. AgroLux competent cell solution: 10% glycerol, 20 mM $CaCl_2$, autoclaved.
2. Agrobacterium cultures: AgroLux cells are grown in liquid LB medium containing 50 µg/mL gentamicin (Gm50) and 25 µg/mL rifampicin (Rif25). AgroLux containing a binary expression plasmid can further be selected with the addition of antibiotics specific to the plasmid (*see* **Note 4**).
3. Leaf infiltration buffer: 10 mM 2-(N- morpholino) ethanesulfone (MES), 10 mM $MgCl_2$, pH 5.7, 100 µM acetosyringone. Prepare fresh preferably, or keep at 4 ^{o}C for short-term storage.

2.5 Software

1. Open source software ImageJ (https://imagej.nih.gov/ij)

3 Methods

3.1 AgroLux competent cells and transformation

This procedure describes how to prepare competent cells of the AgroLux strain. It provides a freeze–thaw method for the efficient introduction of binary plasmids into AgroLux. AgroLux can be used with or without a binary plasmid as bioluminescence is independent of plasmid transformation (*see* **Note 3**).

3.1.1 AgroLux competent cells

1. From an AgroLux glycerol stock, spread bacterial cells onto a LB agar (1.5% agar) plate supplemented with 50 µg/mL gentamicin (Gm50) and 25 µg/mL rifampicin (Rif25). Incubate the plate for 36-48 h at 28 ºC.
2. Pick a single colony from the plate and inoculate it in 3 mL of LB medium with Gm50 and Rif25 antibiotics. Incubate the LB pre-culture at 28 °C, 180 rpm for 24-36 h.
3. Transfer 1.5 mL of the pre-culture to 50 mL of LB medium, supplemented with Gm50 and Rif25, in a 250 ml Erlenmeyer flask. Grow the culture for 12 to 16 hours at 28 °C, 180 rpm until OD_{600} reaches between 0.5 to 1.0.
4. Once the culture as the appropriate OD_{600}, keep the culture on ice for 15 minutes to stop bacterial growth.
5. Centrifuge the culture in a 50 mL falcon tube at 5000 g for 15 min at 4 °C.
6. Discard the supernatant and remove all media using a pipette.
7. Resuspend cells delicately in 1 mL of pre-chilled AgroLux competent cell solution (*see* Materials, **Subheading 2.4.1**), keeping cells on ice the whole time.
8. When the pellet is entirely resuspended, aliquot in 1.5 mL Eppendorf tubes, 50 µL per tube and freeze immediately in liquid nitrogen.
9. Store at -80 °C (*see* **Note 5**).

3.1.2 AgroLux transformation

1. Put a frozen AgroLux competent cell aliquot on ice and wait for it to melt.
2. Add 2 µL of purified plasmids to 50 µL aliquot of AgroLux competent cells and wait 10 min on ice with no agitation.

3. Flash-freeze the cells in liquid nitrogen for a few seconds (*see* **Note 6**).
4. Incubate cells for 5 min at 37 ºC.
5. Add 100 µL of LB medium with no antibiotics and incubate 2 h at 28 ºC with agitation.
6. Spread 150 µL of the LB culture on a plate containing Gm50, Rif25 and the appropriate antibiotics for plasmid selection.

3.2 Agroinfiltration

3.2.1 Bacterial growth and leaf agroinfiltration

1. Inoculate Agrobacteria containing the plasmid of interest in 5 mL of LB plus the required antibiotics (commonly Rif, Gm, Kan) in a 50 mL falcon and grow for 16-24 h at 28 ºC, 180 rpm.
2. Spin cultures down at 2000 g for 10 min at room temperature, centrifuge acceleration/deceleration set to max; discard the medium.
3. Resuspend each pellet in 10 mL of leaf infiltration buffer (*see* Materials, **Subheading 2.4.3**) and measure OD_{600}.
4. Adjust OD_{600} to 0.5 (can be different according to the experiment, *see* **Fig. 1**) in leaf infiltration buffer and add acetosyringone to a final concentration of 100 µM.
5. Incubate cultures for 1 h at 28 ºC and water the plants to strengthen the leaves via turgor and open the stomata.
6. Infiltrate *N. benthamiana* leaves with a needless syringe (*see* **Note 7**).

3.2.2 Post-infiltration incubation conditions

1. *N. benthamiana* plants are kept in a growth cabinet at 22 °C under a 16/8 h light/dark regime, at a light intensity of 30 µmol $m^{-2}s^{-1}$.

3.3 AgroLux luminescence imaging and data analysis

AgroLux bioluminescence signals *in planta* are measured using either a standard charge-coupled device (CCD) camera or a plate reader. CCD cameras have the advantage to be widely available in laboratories.

3.3.1 Whole leaf imaging

1. Detach the agroinfiltrated leaves after 2 to 7 days post-infiltration (dpi) (*see* **Note 8**-**9**).
2. Turn on the Image Quant LAS4000 imaging system and set the program to chemiluminescence (*see* **Note 10**)
3. Set the option Exposition/Resolution to 'High' and select the digitisation option to have a photo of the leaves.
4. Place the leaves with the abaxial side facing the camera (*see* **Note 11**), and adjust the focus using the 'Focus' option.
5. Keep the leaves for 5 min in the dark prior to taking bioluminescence measurements (*see* **Note 12**).
6. Take measurements with 5 minutes exposition. If the signal is weak, exposition time can be set to 10 min or more.
7. Save the image as a *.tiff* file for image analysis.

3.3.2 Image intensity processing using ImageJ

1. Import images (*.tiff* file) into ImageJ (*see* Materials, **Subheading 2.5** and **Note 13**).
2. To set the type of measurement to be made, select *Analyse > Set Measurements...* and select *Area, Mean gray value* and *Min & Max gray value* (*see* **Note 14**).
3. Open the ROI Manager, select *Analyse > Tools > ROI Manager...*
4. Use the appropriate selection tool (Circle or Freehand) to select a region of interest (ROI) (*see* **Note 15**).
5. Click on *Add* in the ROI Manager.
6. Repeat the selection process and add ROIs.
7. When all ROIs are selected, click on *Measure* in the ROI Manager to open the *Results* window.
8. Copy and paste the results into a spreadsheet for further analysis (*see* **Note 16**).
9. Subtract background values from each image based on the average value of non-infiltrated zones on the leaves.

3.3.3 Apply a look-up table (LUT) to colour images using ImageJ

1. Import images (*.tiff* file) into ImageJ to apply a LUT (**Fig. 1**) (*see* **Note 17**).

2. Import LUT, select *File* > *Import* > *LUT*…, find the LUTs subfolder in the ImageJ program folder and select the appropriate LUT (*see* **Note 18**).
3. Scale the image brightness, select *Image* > *Adjust* > *Brightness/Contrast*… and click on *Auto* (*see* **Note 19**).
4. Add a calibration bar, select *Analyse* > *Tools* > *Calibration bar*…
5. Save the image.

3.3.3 Leaf discs data acquisition

1. Add 200 µL water in wells of a 96-well plate.
2. Punch discs from agroinfiltrated leaves (6 mm diameter max) and gently add one leaf disc per well (*see* **Notes 20**).
3. Insert the plate in the Infinite M200 reader (*see* Materials, **Subheading 2.3**), and keep the plate in the dark for 5 min prior to bioluminescence measurements (*see* **Note 12**).
4. Set the plate reader to luminescence measurement with an integration time of 1 sec.
5. Save the data in a spreadsheet for further analysis.

4 Notes

1. *N. benthamiana* plants are widely used by plant molecular biologists to transiently express genes by agroinfiltration [11]. AgroLux can be used to monitor Agrobacterium activity in various plant species that are suitable for agroinfiltration [12].
2. AgroLux (unique ID RSPJ07) is a bioluminescent derivative of *Agrobacterium tumefaciens* strain GV3101 that carries a single-copy genomic integration of the *luxABCDE* operon driven by the constitutive *nptII* promoter [9]. The strain is available upon request.
3. AgroLux can be used as a bioluminescent reporter post-agroinfiltration by mixing other Agrobacterium cultures carrying expression vectors or as a recipient of binary plasmids of interest [9].
4. Common binary expression plasmids have a kanamycin resistance gene and can be selected by supplementing the LB medium with 50 µg/mL kanamycin (Kan50).

5. Before using freshly prepared AgroLux competent cells, it is a good practice to test an aliquot. Spread the bacterial cells on different plates containing common antibiotics, such as kanamycin (Kan50), streptomycin (Strep50) or carbenicillin (Carb100). AgroLux cells should not grow on any plate containing antibiotics, except for gentamicin (Gem50) and rifampicin (Rif25).
6. AgroLux can also be transformed through electroporation. See ref. [13] for alternative methods for *A. tumefaciens* transformation.
7. The youngest, fully expanded leaves of 4-6-week old *N. benthamiana* are easy to infiltrate from the abaxial (lower) side with a needle-less syringe and cause a good transient protein expression. When co-expression with P19 silencing inhibitor [14], mix the culture containing the binary plasmid encoding P19 with the culture containing the gene of interest (normally 1:1) prior to infiltration.
8. For transient protein expression, it is recommended to harvest plant tissue at 5 dpi. For imaging bioluminescence of AgroLux, bioluminescence can be monitored throughout 7 dpi and more.
9. If images are not taken within 30 min after the leaves have been detached, it is recommended to keep the detached leaves in a plastic box with a wet tissue to maintain humidity high.
10. The tray position of the imaging system can be adjusted to measure 2 to 6 leaves in one image.
11. Bioluminescence levels are stronger when leaves are imaged from the abaxial (lower) side where they have been infiltrated.
12. Keeping the leaves in the dark before bioluminescence measurements reduces the background coming from the chloroplasts.
13. ImageJ allows image pixel quantification. It is recommended to follow the Image Intensity Processing protocol from ImageJ documentation (https://imagej.nih.gov/ij).
14. When setting measurements, tick the relevant boxes. *Min & Max gray value* shows the background (Min) and if pixels are saturated (Max).
15. Alternatively to the selection tools, select *Image* > *Adjust* > *Threshold*… and adjust the pixel thresholds to highlight only the ROIs. Then, use the *Wand (tracing) tool* to select ROIs and add your selections to the ROI Manager.
16. Use the *Mean values* for selected ROI intensities.

17. Applying a LUT allows to display grey values as colourised pixels. The colourised pixels in the pseudo-coloured image reflect differences in pixel intensity.
18. To determine the appropriate LUT, select *Image* > *Color* > *Display LUTs*… to open the look-up tables.
19. To compare multiple images, select *Set* to adjust the brightness with specific values.
20. It is important that leaf discs are floating at the top of the water and are not submerged. Leaf discs should be placed with the abaxial side up to obtain the brightness signals and reduce variability.

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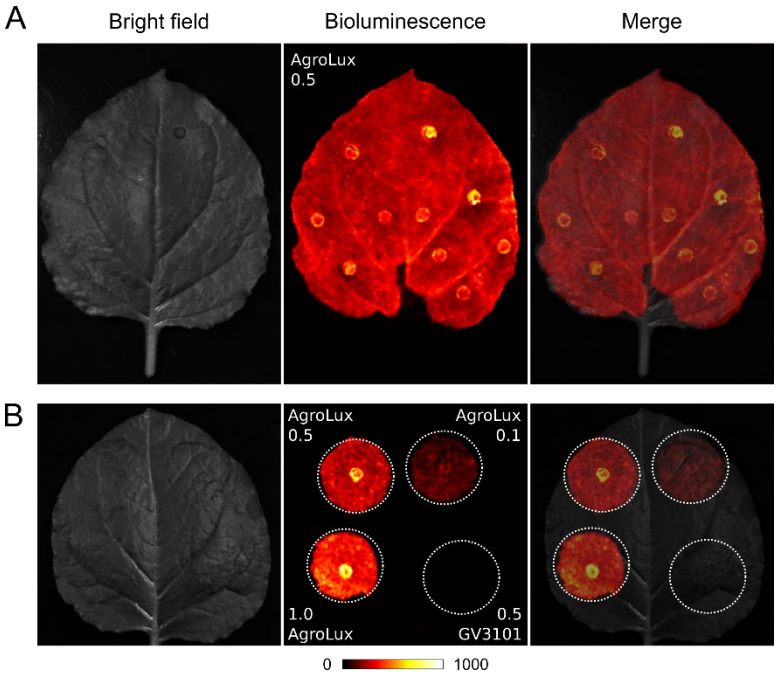

Figure 1 *In planta* imaging of AgroLux bioluminescence.

(**A**) The whole *N. benthamiana* leaf was agroinfiltrated with AgroLux at OD_{600} 0.5 using a needless syringe. The infiltration sites are visible as brighter round prints. (**B**) AgroLux was spot-infiltrated with various optical densities (OD_{600} = 0.1; 0.5; 1.0) (*see* [9]). Wild-type Agrobacterium GV3101 strain was infiltrated at OD_{600} 0.5 and used as a control. The bioluminescence images were generated at 2 dpi with a CCD camera. A Red-Hot LUT was applied using ImageJ to colour bioluminescence signals.