Chapter 1

Analysis of Plant Root Gravitropism

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Abstract

Gravity is a powerful element in shaping plant development, with gravitropism, the oriented growth response of plant organs to the direction of gravity, leading to each plant's characteristic form both above and below ground. Despite being conceptually simple to follow, monitoring a plant's directional growth responses can become complex as variation arises from both internal developmental cues as well as effects of the environment. In this protocol, we discuss approaches to gravitropism assays, focusing on automated analyses of root responses. For *Arabidopsis*, we recommend a simple 90° rotation using seedlings that are 5–8 days old. If images are taken at regular intervals and the environmental metadata is recorded during both seedling development and gravitropic assay, these data can be used to reveal quantitative kinetic patterns at distinct stages of the assay. The use of software that analyzes root system parameters and stores this data in the RSML format opens up the possibility for a host of root parameters to be extracted to characterize growth of the primary root and a range of lateral root phenotypes.

Key words *Arabidopsis thaliana*, Gravitropism, Gravity perception, Root tip, RSML, Time-lapse photography

1 Introduction

Land plants use the gravity vector as a guide for directional growth of their organs, i.e., exhibit gravitropism. At its simplest level, roots grow downward into the soil, improving anchorage and water and nutrient uptake, whereas shoots grow upward to access light for photosynthesis, exchange gases, and contribute to reproduction. However, these descriptions hide a vast array of more subtle behaviors and interactions. Thus, roots and shoots do not simply grow downward or upward but show complex kinetics of response over time from, e.g., almost no gravitropic response in the young root (e.g., [1, 2]) to the development of complex root system architectures where organs maintain characteristic angles to the direction of gravity, their so-called gravitropic setpoint angles [3]. These setpoints can differ between adjacent organs and can change through-

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out development. The observation of negative gravitropic curvature in shoots and the positive gravitropic growth of roots implies that each organ possesses gravity-sensing machinery that allows it to identify changes in its orientation and respond by redirecting growth [4, 5]. However, signals other than gravity interact with gravitropism to fine-tune these responses. For example, shoots show strong positive phototropism to blue light, which can often override purely gravitropic responses [6]. In roots, however, gravitropism tends to be among the most significant of these directional growth responses, and competing tropic pathways triggered by, e.g., light, touch, water gradients, salt, and/or oxygen tend to modulate the effectiveness of gravitropism when their contribution is needed most [7]. The complexities of the kinetics of tropic responses, which are driven by both developmental changes and a plethora of environmental interactions, require the use of methods to precisely quantify the growth rates of organs, their angles, and how these change over time in order to dissect the relative contributions of these multiple factors.

Many different organs from diverse species and ages of plants have been used to investigate gravitropism, so in this protocol, we will focus on the analyses of the seedling root as a widely used model for characterization. There have also been many different methods used to create gravitropic stimuli, including rotation of the plant through 90°, 135°, or 180°, degrees; however, the important and consistent feature of these experiments is that the plants are rotated beyond the 40° "tipping point" angle [8], which represents a threshold for triggering robust gravitropic reorientation. For many studies, a 90° reorientation is a standard approach that is especially convenient if you are growing samples in containers, such as square Petri dishes. Although an endpoint measurement of the root tip angle after a period such as 12 h of gravitropic response has been traditionally used to characterize responses, this approach misses many of the key characteristics of the fine-scale spatial and temporal structure of the gravitropic response. Thus, modern analyses use time-lapse photography of root growth and curvature in response to gravistimulation to quantify angles and growth rates with high temporal resolution. Some gravitropic phenotypes are observed only in the early or late stages of gravity perception, providing important insights into the interplay between cell division, cell elongation, and differentiation, which are lost with a single timepoint measurement. Therefore, in this protocol, we will describe time-lapse image analysis of gravitropic response, concentrating on factors to allow for robust reproducibility and comparison between experiments. In addition, we will discuss the software approaches that facilitate automated and semiautomated analyses and that generate outputs designed to promote data sharing and reanalysis. Indeed, some uniformity in the approaches being applied across disparate studies is now allowing for much

more robust comparisons between experiments, and so we will describe one such standardized approach built around the Root System Markup Language (RSML) applied to the analysis of root tropic response.

2 Materials

2.1 General Materials

1. Sterile, square, non-gridded 100 mm Petri dishes.
2. Chemicals to make solid growth medium: e.g., Phytagel and sucrose (*see* **Note 1**).
3. Linsmaier and Skoog (LS) nutrient salts.
   Make up ½ strength LS medium; add 10 g/L Phytagel and 3g/L sucrose (optional, plants will grow faster); autoclave; and allow to cool to a temperature allowing safe handling. Pour 30 mL/square Petri plate, replace the plate's lid, and allow to cool to room temperature. Place in a plastic bag and tape shut. Can be stored in a fridge for several weeks.
4. 70 % (v/v) ethanol.
5. 1.5 mL microfuge tubes
6. Autoclaved filter paper discs, ~ 3 cm diameter, kept sterile.
7. Medical micropore tape.
8. Sterile either 200 μL pipette tips, tweezers, or toothpicks (for planting seeds, see **step 3.1.3** below).
9. A standard controlled growth environment such as a growth chamber with the following conditions:
   a. Light; 100–225 μmol/m^2
   b. Temperature: 21–22 °C
   c. Humidity: 65–90%
   d. Controlled photoperiod. Depending on the experimental design, this could be constant darkness, constant light, or a photoperiod such as 12-h day/12-h night or 16-h day/8-h night (long day) photoperiod. For *Arabidopsis* seedlings, we typically grow them under constant light for 5–7 days prior to use.
   e. Note the time of day when you start the experiment (*see* **Note 2**).

2.2 Options for Image Acquisition

There are many options for recording the images for gravitropic analyses. All work well for this application as all can pass high-resolution images to the image analysis software. We list below some popular alternatives:

1. Flatbed scanner (must have at least 300 dpi, *see* **Note 3**)
2. Smartphone

3. Dissecting microscope
4. Digital SLR camera
5. PiCam (>=5Mega Pixel, *see* **Note 4**)

3 Methods

3.1 Preparing Plant Materials

The following is a protocol designed for monitoring root growth in seedlings of the model plant *Arabidopsis thaliana*, but similar protocols are applicable to a wide range of plant species. When designing the experiment, at a minimum, image plants before and after a fixed time of gravitropic stimulus, such as 4 h. However, important quantitative phenotypes can be found using time-lapse photography, so continuously following the plants over 9–12 h is strongly recommended (*see* **Note 5**). An overview of the approach is shown in Fig. 1. These protocols use plants growing under sterile conditions, so all protocols up to **step 4** should be performed in a laminar flow hood or equivalent sterile environment.

1. Surface sterilize seeds in a 1.5 mL microfuge tube, shaking for ~2 min with 70% (v/v) ethanol (*see* **Note 6**). Pour seeds onto sterile filter paper and wait for ethanol to evaporate.

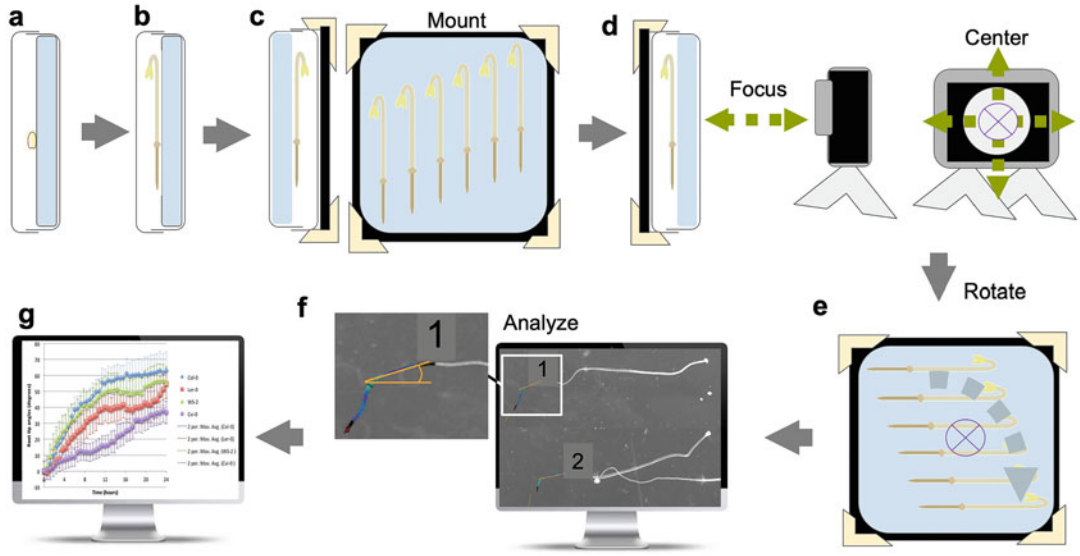

Fig. 1 Different stages of a gravitropic assay for *Arabidopsis thaliana*. (**a**) Seed stratification and germination. (**b**) Growth and development ~5–14 days. (**c**) Petri dishes can be inserted into custom supports with a consistent high-contrast background to aid in subsequent image analysis. (**d**) Camera focused and centered on sample. (**e**) Sample is rotated 90°. (**f**) Images imported into RootTrace automated analysis software. (**g**) Graphical output showing comparative kinetics of reorientation and growth rate

2. Sow six to eight seeds in a horizontal line on plates containing 1 % (w/v) Phytagel, 1/2 strength LS salts, and 0.3 % (w/v) sucrose (or MS salts, *see* **Note 7**).
3. Place the seeds on the surface of the gel using a sterile pipette, sterile tweezers, or an autoclaved toothpick (*see* **Notes 8** and **9**).
4. Store the plates at 4 °C for 3 days in darkness to vernalize seeds. Vernalization aids in synchronous germination once exposed to light/room temperature.
5. Place plates vertically in the controlled environment for 4 d (or until they reach the desired developmental stage) (*see* **Note 10**).
6. Insert plates into a Petri dish holder in front of the camera with plants aligned vertically. Optimally, to reduce possible effects of phototropism, subsequent steps related to imaging the gravitropic response should be conducted in darkness using either infrared or dim green light for illumination (*see* **Note 11**). Rotate plates 90°, and set the camera to photograph at desired intervals for the chosen length of time (*see* **Notes 11** and **12**).
7. Collect images (*see* **Note 13**).
8. Measure images using PlantCV [9] or semiautomated image analysis methods such as RootTrace [10] or AstroDart/archiDART [11]. If using ImageJ/FIJI [12], then the SmartRoot package [13] or RootNav software [14] allows saving of data in the Root System Markup Language (RSML) format [15] (*see* **Note 14**).
9. Plot data using a spreadsheet or graphing software such as Microsoft Excel or the R-Shiny AstroDart package (*see* **Note 15**).

3.2 Example Data Processing Through SmartRoot and AstroDart

Wild-type *A. thaliana* (Col-0) was grown and imaged using a PiCam as described above, with the plants growing in a vertical orientation (defined as 0° in subsequent analyses). The plate was then rotated through 90° for gravitropic stimulation (GS) or left with the plants oriented vertically at 0° for no gravitropic stimulus (NGS). Growth was photographed every 15 min for 24 h. This represents a degree of oversampling for *Arabidopsis* as 1-h timepoints provide adequate resolution to follow most gravitropic reorientation kinetics. However, the 15-min frequency provides increased resolution should a particular part of the response be identified as needing more in-depth scrutiny from the initial analysis. Images were collected as ".tif" files and converted to black and white for subsequent image analysis (*see* **Note 16**). Roots were analyzed at 1-h timepoints for the first 20 h using the SmartRoot and AstroDart software applications to look at root curvature and growth rate.

SmartRoot Processing

1. Open the SmartRoot Explorer plugin through ImageJ. Select "Display Axis" and "Display Nodes" (Fig. 2a).
2. In the SmartRoot Explorer window, navigate to the location of the images (Fig. 2a; for multiple genotypes in an image, *see* **Note 17**).
3. Open the first image and use the orange "trace root" tool to trace main roots, clicking to add nodes and right clicking to end the root. The green tracing tool can be used to add lateral roots. When the image is closed, a RSML file is automatically generated in the same folder and with the same name as the image.
4. Open the next image. Right click and select file—import previous data file to upload the previous RSML annotation. Drag the existing nodes to continue tracing the root (Fig. 2b; *see* **Note 18**).
5. Repeat tracing with each image in sequence.

AstroDart Processing

1. Once all RSML files are generated, they need to be renamed to follow a pattern for AstroDart to be able to read them. Genotype, time, treatment 1, treatment 2, and replicate# are the available options in AstroDart and can be placed in any order in the file name with a "_" dividing them. Only desired options need to be added (*see* **Note 19** for example of file naming).
2. RSML files to be processed are then placed in a folder together.
3. AstroDart is launched through R, and RSML files are uploaded by entering the locations using their file navigational pathway on your computer.
4. The files are indexed by entering the position of genotype, time, treatment 1, treatment 2, and replicate# locations within the RSML file name.
5. Process data. Once finished, the headings along the top bar provide data analysis options. There is also the possibility to download the processed data as a spreadsheet file for further data analysis. The available analysis packages are as follows:
   (a) archiTect: Contains many quantified features of the root system including root curvature (mean apex orientation of first-order roots; Fig. 3a) and growth rate (Fig. 3b). Selected variables can be plotted, and boxplots are available with a slider bar to allow for visualization of the distribution of data at each timepoint (e.g., Fig. 3c for root tip angle).

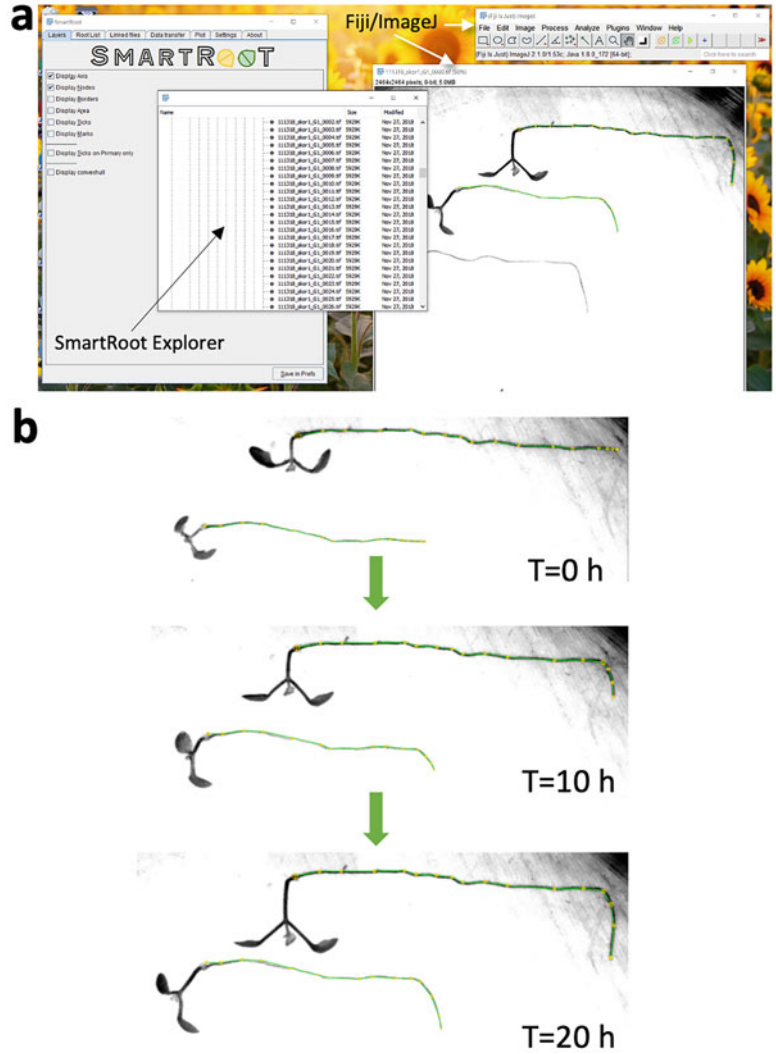

Fig. 2 Example image and data processing through SmartRoot. (**a**) SmartRoot user interface with "Display Axis" and "Display Nodes" selected. SmartRoot Explorer allows user to define the file pathway to black and white images. The Fiji/ImageJ analysis window is also shown with an opened image, where root tracing with the orange Trace option for main root is in operation. (**b**) Example of consecutive tracing of roots at timepoints 0, 10, and 20 h post-gravistimulation made by altering the position of nodes in the ImageJ tracing to follow root growth and curvature

(b) archiDraw: Shows an overlay of the traced lines of the roots for visual comparison of all roots for each treatment (Fig. 4a).

(c) archiAngle: Plots the angle distributions for each treatment (Fig. 4b).

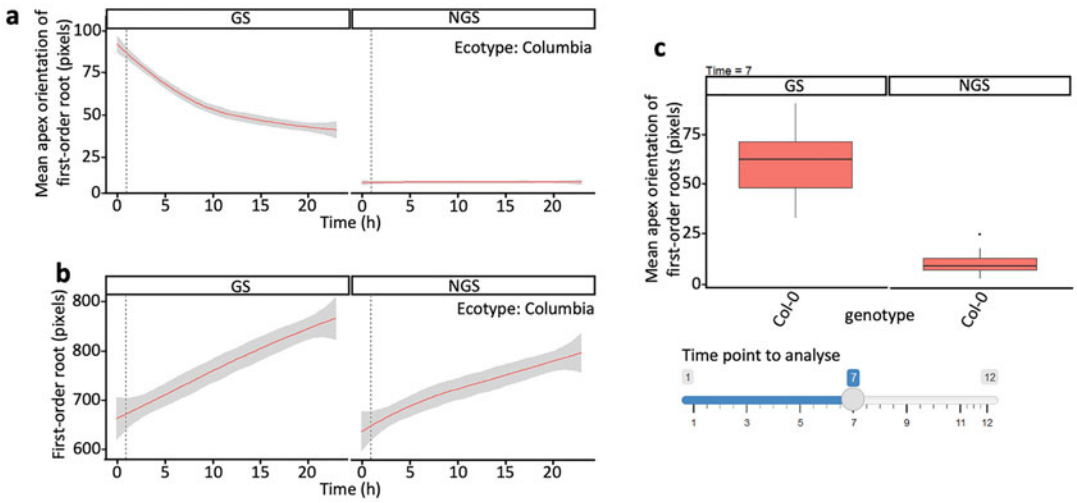

Fig. 3 Output of AstroDart archiTect showing (**a**) mean apex orientation of first-order roots (i.e., main root curvature) comparing roots with gravitropic stimulus (GS) and no gravitropic stimulus (NGS). (**b**) AstroDart archiTect graph of growth rate between GS and NGS roots. (**c**) Boxplot of mean apex orientation of first-order roots showing the use of archiTect slider bar. Example is set at timepoint 7-h post-gravistimulation

4 Notes

1. Although we suggest Phytagel as the gelling medium to provide a solid surface for the plant to grow on, other researchers have successfully employed agar, Gelrite, or even gelatin to replace Phytagel in these kinds of experiments.
2. Time of day can have dramatic effects on plant responses. Therefore, it is important to conduct analyses at the same time of day and record this data so that other researchers can make more robust comparisons to your experimental results.
3. Most flatbed scanners will work for this application. Important parameters to check are (i) the scanner can operate while mounted vertically (to avoid having to lay the Petri dishes flat for each timepoint and so applying regular transient reorientation to the samples just to be imaged), (ii) it can focus on your samples, and (iii) the illumination from the scanner is not so intense as to stress the plants. This is readily checked by growing a test set of plants vertically with and without scanning and monitoring whether growth is inhibited or whether the leaves begin to accumulate the bluish pigment anthocyanin, which is a marker for stress.
4. PiCams (https://www.raspberrypi.org/products/camera-module-v2/) are inexpensive camera modules that work with the Raspberry Pi minicomputer. The video output from the Raspberry Pi can then be fed into the analysis software running, e.g., on a PC.

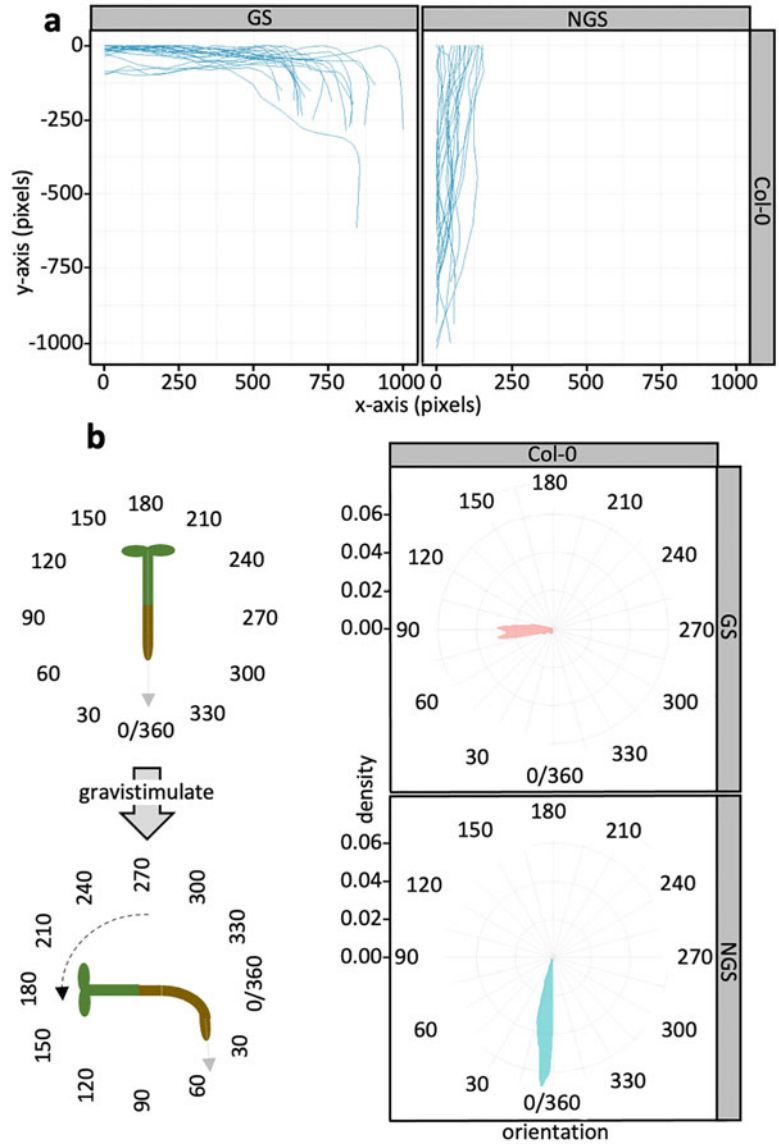

Fig. 4 (**a**) Overlay of tracing of all analyzed roots visualized at the 20-h timepoint post-gravistimulation using AstroDart and archiDraw. (**b**) Summary of all root trajectories measured for GS and NGS roots in AstroDart using archiAngle

5. When selecting your interval rate, consider your species growth rate, size, camera resolution, and hard drive space. For *Arabidopsis*, taking a photo at 300 dpi every 0.5–1 h for ~9 h is usually sufficient to acquire gravitropic phenotypes. However, some other plant varieties or some *Arabidopsis* mutants reveal interesting nastic movements if photographed at 15-min intervals or may take longer than 9 h to reach a stable gravitropic

setpoint angle. It is important to run experiments until this stable angle is achieved to characterize the complete gravitropic response. The following are factors to consider and record:

(a) Resolution of the images (e.g., 300 dpi).

(b) Duration of imaging (6–24 h).

(c) Frequency of imaging (15–30 min; more frequent imaging generates higher resolution time course data, but this may have to be adjusted depending on available storage space).

(d) File format (jpg & tif). Although jpg is a lossy compression format (i.e., it loses some data during image compression for storage), the resolution of the images for tropic analyses is usually low enough where lossy compression has little impact on the quantitative results. However, for the most faithful representation of response, store data using a lossless compression format, such as a lossless tif.

(e) Location for saving data. This should include plans for both the short and long term. Local storage is highly appropriate for initial analyses, but it is important to have plans for long-term storage of potentially large amounts of imaging data. Cloud-based storage such as with CyVerse [16], or alternative commercial options, provides a convenient and robust longer-term storage and backup option. Forethought in file naming conventions is also important to aid in future data retrieval and possibilities for reanalysis (*see* **Note 13**).

6. If microbial contamination occurs, try changing the seed sterilization method by rinsing with 70 % (v/v) ethanol three times, and then allow to air-dry onto sterile filter paper. Or follow the ethanol treatment with either the following:

   (a) Bleach treatment:

      (i) Pour off 70% (v/v) ethanol and add ~1 mL bleach solution (10 mL sodium hypochlorite/bleach, 40 mL H_20, and 50 μL sodium dodecyl sulfate), shake for 10 min, rinse three times with 70 % (v/v) ethanol, and then continue with protocol.

   (b) Chlorine gas (vapor-phase) sterilization:

      (ii) Vapor-phase sterilization uses chlorine gas and so should only be performed with adequate ventilation/in a fume hood.

      (iii) The vessel for the sterilization should be ~ 1 L in volume, chlorine resistant (e.g., glass), and able to be sealed; usually a desiccator jar is used.

(iv) Place seed in open microcentrifuge tube in a rack inside the desiccator jar alongside 100 mL bleach in a 250 ml glass beaker.

(v) Add 3 mL concentrated HCl to the bleach and seal the jar.

(vi) The resulting chlorine gas will sterilize the seeds in 6–12 h. Longer treatment times may lead to some drop in seed viability.

(vii) Move jar to laminar flow hood, and under sterile conditions, remove microfuge tubes and close their lids.

7. There are several basal salt media used to grow *Arabidopsis*. In addition to Linsmaier and Skoog, Murashige and Skoog is also widely used. Use at only ½ the recommended concentration as full strength of either of these media will stress the plants and lead to slow and distorted growth.
8. Seeds can be sown in parallel lines or offset to make diagonal lines to reduce the likelihood of root collisions where the root of one responding plant grows into that of the one next to it during its gravitropic response (see Fig. 1c). For picking up and planting small seeds like *Arabidopsis*, surface tension on a damp sterile toothpick can be enough to hold the seed, but with larger seeds like cotton, tweezers can be used. If working with larger seeded monocot's like *Brachypodium*, rice, or corn, submerge the end of the kernel so the radicle will emerge within the gel matrix of the medium but near the air/gel interface.
9. When sealing the plates, use medical micropore tape before removing them from the laminar flow cabinet to prevent possible microbial contamination.
10. Plants can be grown under a range of lighting regimes as appropriate for each specimen. Record the photoperiod, light quality, and quantity during seedling development as key metadata for each experiment.
11. When providing the gravitational stimulus and following the response, provide a dark growth environment to reduce phototropic effects. Alternatively, low levels of diffuse green light can be used, or if you have infrared cameras, then infrared illumination during imaging of response is a preferred option. Gravitropic phenotypes can still be detected in environments with background white light illumination, such as in a standard growth chamber, or when using intermittent white light illumination for the photography, but it is important to remember there will potentially be phototropic growth superimposed on any gravitropic response. In addition, minimizing mechanical

stimulation when handling the plates is essential as mechanical signaling is known to interact with the gravitropic response network to alter root growth [17].

12. When imaging samples, ensure they are parallel to the edge of the scanner or camera (to avoid the need to realign them in software later).
13. Some care in optimizing image contrast and clarity at this stage can make it easier to measure roots later on. It is also important to adopt a naming convention for your files such as with date and sample name to ensure ease of retrieval. Adding metadata about genotypes, age, and important environmental conditions can also be extremely useful and also allow for automated searching for datasets for different characteristics in the future. For example, for an experiment using the Col-0 ecotype at 5-d post-germination, grown under purple light (blue and red LEDs), with an image every 15 min, this data can be encoded in the file name as Genotype_age_treatment_timedelay_image#
    Col-0_5dpg_purplelight_15min_001,
Col-0_5dpg_purplelight_15min_002,
Col-0_5dpg_purplelight_15min_003, etc.
14. First measure the root tip angle before the plants were gravistimulated. Then, measure the new orientation that the root takes in every image.
    (a) Software for image processing
        (i) RootNav (WinOS) [14]; https://www.nottingham.ac.uk/research/groups/cvl/software/rootnav.aspx
        (ii) RootTrace (WinOS) [10]; https://www.nottingham.ac.uk/research/groups/cvl/software/roottrace.aspx
        (iii) FIJI/ImageJ with SmartRoot plugin [13]; https://www.quantitative-plant.org/software/smartroot
        (iv) Python using the PlantCV [9] or RootNav2 library [18]; https://plantcv.danforthcenter.org; https://github.com/robail-yasrab/RootNav-2.0
15. Software for data visualization of RSML formatted datasets
    (a) RootNavPortal: https://github.com/Chagrilled/RootNavPortal
    (b) RootTrace R-shiny: https://rmtrane.shinyapps.io/RootTrace/
    (c) AstroDart is an R-shiny application designed for rapid visualization of root system architecture (RSA) data using the ArchiDART package [11]; https://www.quantitative-plant.org/software/archidart. To use this with your own data, you will need the location of the folder containing all the RSML files that contain your root data and then follow the application's instructions.

(i) install R-studio

(ii) library(shiny)

(iii) shiny::runGitHub("archidart/astrodart", "astrodart")

16. For SmartRoot to accurately process images, they must be in black and white. Color can be readily removed in a batch process in, e.g., Adobe Photoshop.
17. Only one genotype per image can be processed at a time. If there are multiple genotypes per image, then the image must be processed individually for each genotype and renamed accordingly.
18. If more nodes are needed, while the root trace tool is selected, right click and select "Multiply Nodes."
19. For example, Col-0_GS_4_R1 indicates the following:
    (a) Genotype: Col-0
    (b) Treatment 1: GS
    (c) Time/Day: 4 (Time is always listed as "days," but as long as units are kept consistent in naming process, any unit can be used)
    (d) Replicate: R1

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