Automated Detection of Germinated *Arabidopsis thaliana* Seeds in Microplates

**Introduction**

The success of seed germination and seedling establishment determines crop productivity, which has major social and economic implications considering that plant seeds are the main source of human calories. Seed germination results from the integration of internal signals and environmental factors in order to secure progeny survival. Genetic factors together with storage conditions determine the potential for rapid and uniform emergence and development, referred to as seed vigor.¹

Major advances have been achieved in seed biology research, leading to the identification of key genetic regulators of seed development and germination. The success of these studies relies on the extensive genetic resources available for the plant model *Arabidopsis thaliana* (https://www.arabidopsis.org). Far from behaving homogeneously, seeds within a population that display natural differences can compromise crop synchronicity and yield. Nonetheless, the molecular mechanisms associated to seed variability are far from being understood, as technically challenging approaches are required.

Seed germination studies rely on the determination of embryo radicle emergence as readout of seed viability. These analyses are performed by visual observation of the seeds over a period of time that varies depending on the seed genotype and germination conditions. The current methodology for seed germination analysis is very laborious and it hinders high-throughput combinatorial studies where multiple seed variants and germination conditions could be assessed. The development of an automatized quantification of seed germination will allow multifactorial studies in a timely manner, which could contribute to provide novel solutions for securing crop productivity in the face of increased adverse environmental conditions.
EnSight® is a multimode plate reader that can be equipped with a well imaging module to image 96- or 384-well plates. Images can be acquired both in brightfield and fluorescence modes, using five LED and four fluorophores. Each image per well is acquired at 4X magnification, using a laser-guided autofocus, allowing fast acquisition time. Image acquisition and online-analysis are performed by the software, Kaleido, which offers preset, yet customizable, analysis tools. Well imaging is habitually used for cell-based assays, for instance to quantify cell number, confluency and average fluorescence intensity.

In this application note, we assessed the possibility to detect the germination of Arabidopsis seeds using the well imaging module of EnSight. For such unconventional application, a new Kaleido algorithm was created to analyze images; the algorithm allowed seed detection and classification as germinated, according to radicle emergence, or non-germinated ones. Germinating radicles were automatically detected at very initial stages, thanks to their endogenous blue fluorescence.

In order to assess the ability of the algorithm to detect variations of seed germination at different time points after seeding, seeds were incubated with different concentrations of the plant hormone abscisic acid (ABA) or NaCl to confer salinity stress.

The algorithm successfully calculated the fraction of germinated seeds over the total amount of seeds present in each well of the microplate, allowing the analysis of kinetics and dose-response data.

**Material and Methods**

**Material**
Black CellCarrier plates with transparent bottom (PerkinElmer, # 6005550) were used for seeds growth, treatment and images acquisition. Arabidopsis Col-0 seeds were obtained from Nottingham Arabidopsis Stock Centre (NASC). Other reagents used in this application note are: NaCl (PanReac 131659), ABA (Sigma 862169), plant agar (Duchefa Biochemie P1001) and autoclaved pure water (MilliQ).

**Seeding Protocol**
A. thaliana seeds were resuspended in 0.030% plant agar at a final concentration between 4 - 6 mg seeds/ml agar. 60 µl of suspended seeds were manually pipetted into each well, using blunt tips. By using this suspension, about 20 seeds per well were seeded in each well.

**Seed Treatment with NaCl**
NaCl stock solution was prepared in pure water at 5 M concentration. Seeds were suspended with agar supplemented with NaCl to reach a final NaCl concentration equal to 200, 100, 75, 50, 25 or 10 mM. Control wells were incubated with agar only, as control. Six replicates per conditions were seeded, corresponding to six different wells.

**Seed Treatment with ABA**
Abscisic acid (ABA) was prepared in methanol at 50 mM concentration. Seeds were suspended with agar and ABA stock solution in different proportion, to reach final ABA concentration equal to 5, 2, 0.8, 0.32, 0.13, 0.05, 0.02 and 0.01 µM. Control wells were incubated with agar only, as control. Six replicates per conditions were seeded, corresponding to groups of six different wells.

**Image Acquisition**
EnSight was positioned in a greenhouse to control germination conditions set at 22 °C, RH of 50-60% with a 14-hr light/10-hr dark cycle and light intensity 300 µmol/m²·s. Immediately after seeding A. thaliana seeds, the plate was loaded in EnSight in order to automatically acquire images using a Kaleido protocol set to automatically switch between incubation and image acquisition steps. More in details, both brightfield and fluorescent images were acquired to detect seeds and germinating radicles, respectively. Brightfield images were acquired with 5% excitation power, 4 ms of exposure time and 160 nm focus offset. Fluorescence images were acquired using the UV LED for excitation at 385 nm, using 100% excitation power, 30 ms exposure time and three offsets: 110, 200 and 280 µm offsets. These acquisition settings are summarized in Table 1.

The energy density corresponding to UV irradiation can be estimated to < 0.6 W/cm² at 385 nm; this irradiation corresponds to an exposure of noon sunlight of only 22 s in central Europe2,3, if the spectrum of the sun would be restricted to the same narrow transmission band used in the EnSight. Therefore, it is reasonable to exclude seeds damages or stimulation due to UV images acquisition.

Imaging conditions were the same throughout all experiments. The four-hours incubation steps were performed outside of the reader (an option available as default on EnSight), in order to expose the plate to the greenhouse environment, including light and temperature.

**Data Analysis Using Kaleido**
A dedicated assay specific analysis method was prepared and imported in Kaleido. The method detected and counted non-overlapping seeds in brightfield images; the underlying algorithm takes advantages of properties related to seeds texture and roundness. The fluorescence channel is then used to detect germinating radicles. In fact, Arabidopsis roots are characterized by endogenous fluorescence caused by cell walls (Grossmann 2018).4 Germinating seeds are defined by a simple threshold in the UV channel, which defines the minimum fluorescent area fraction of the seed, that corresponds to the germinating radicle (Figure 2). We arbitrarily set a threshold value of 0.1, i.e. we considered those seeds as germinated with a fluorescent area ≥ 10% of the total area of the seed.

<table>
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<th>Specification</th>
<th>Brightfield Images Acquisition</th>
<th>Fluorescent Images Acquisition</th>
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<tr>
<td>Excitation Filter</td>
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<td>Excitation Power</td>
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<td>Exposure Time</td>
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<tr>
<td>Focus Offset</td>
<td>160 µm</td>
<td>110, 200, 280 µm</td>
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A set of parameters for seed detection and classification can be tuned by the EnSight user, in order to adapt to experimental variations, such as the dimensions of seeds, the contrast of acquired images and also the threshold for discerning germinating from non-germinating seeds.

For each well, the percentage of germinating seeds was exported by Kaleido as .xml file for further analysis in Excel (Microsoft) and MyAssays® Desktop (MyAssays Ltd.), as described in the result section. A set of additional output parameters of the analysis method (such as the average size and roundness of the two populations of seeds) can also be exported, if desired.

Data Analysis Using MyAssays® Desktop
The values present in the .xml file exported from Kaleido were imported into MyAssays® Desktop. The plate scheme was configured to group replicate samples present in each assay. For each time point, averaged values were computed by MyAssays® Desktop by applying the "XY replicate average" transformation. Non-linear (4PL) fitting was applied using the "XY Fit" transformation on averaged values, to calculate the point of inflection (parameter c), the slope at the point of inflection (parameter b) and the maximum percentage of germination (parameter d).

For each group of replicate samples, the interpolation window started from the first data point (t0) and finished at the time point showing the maximum percentage of germination, in order to avoid bias due to the presence of long radicles, as explained in the Results section.

Results
Arabidopsis seeds are clearly visible in both brightfield and fluorescent modes; the field of view of EnSight covers the vast majority of the well area, allowing the visualization of most of the seeds present in each well of the microplate.

Examples of brightfield and fluorescence images are shown in Figure 1 for a representative well containing untreated seeds of A. thaliana scanned every four hours from time 0 to 32 hours (nine time points).

The brightfield channel shows opaque structure (Figure 1A), while the fluorescence channel shows a widespread fluorescence emitted by seeds, that reveals a characteristic “dotted” surface (Figure 1B and 1C). When germination occurs, the radicle is visible at higher fluorescence intensity, as highlighted by the white color in Figure 1B and 1C.

No germination is expected at time 0, while starting from 16 hours, some seeds show signs of germination; at later time points, radicles are evident both in brightfield and fluorescent images.

The Kaleido software estimated the number of non-overlapping seeds visible in each well and discerned germinated seeds from non-germinated ones. An example of such classification is visible in Figure 2.

This analysis is quantitative and optionally yields different output parameters, such as seed size and roundness; however, the primary output is the percentage of germinating seeds over the total number of seeds detected in each well of the plate. An example of percentages of germinated seed calculated by Kaleido is reported in Figure 1D.

<table>
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<th>8 h</th>
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Figure 1. Examples of brightfield and fluorescent images of a well containing A. thaliana seeds, acquired at different nine time points after seeding. A) brightfield channel, B) fluorescent channel, C) zoom on a specific seed in fluorescent channel, showing radicle emergence, D) percentage of germinated seeds calculated by Kaleido software.
Delay of seed germination upon seed incubation with NaCl

Germination was inhibited by incubating seeds with six increasing concentration of NaCl (10-200 mM), to assess the ability of the algorithm in detecting variation of germination rates. For each condition, seeds were placed in six replicate wells. Controls wells contained untreated seeds. Images were automatically acquired by EnSight for 24 time points every four hours, for a total time of 92 hours. This time frame was considered enough to evaluate possible variations of germination rates.

By comparing images of untreated seeds (Figure 3A) and seeds treated with 200 mM (Figure 3B), it is evident that the highest concentration of NaCl inhibited the germination process for at least 48 hours.

Figure 4 shows the average percentage of germination obtained for untreated and treated seeds.

Notably, almost 100% of germination was obtained 36 hours after seeding of untreated seeds; conversely, only ≈60% of the seeds treated with 200 mM NaCl germinated at the latest time point of this assay (92 hours).

In untreated wells, long seed radicles are evident 48 hours after seeding; such long radicles can cause bias in the data, since they can overlap with other seeds and hamper their detection. Since untreated seeds reached the maximum percentage of germination at the 10th cycle, data interpolation was limited to this final data point. The same procedure was followed for seeds treated with the different concentration of NaCl.

The averaged percentage of germination of untreated wells was fitted by a 4PL regression curve (Table 2). The point of inflection indicates the time required for 50% of seeds to reach the max germination rate; for this reason, we named it G_{50}. For untreated seeds, G_{50} corresponds to 22.2 hours of incubation (6.53 cycles).

The values of G_{50} obtained from seeds treated with the six different concentrations of NaCl are reported in Table 2. A dose-dependent shift is evident from 22.2 hours to 81.5 hours, for 200 mM NaCl, indicating that increasing concentration of NaCl delayed the germination process.

The delay of germination is reflected also by the decreasing “slope” parameter in Table 2, that indicate flatter sigmoidal curves for high concentration of NaCl, as compared to untreated seeds.

The maximum percentage of germination was higher than 90% at all tested concentration of NaCl.

Figure 3. Example of images acquired in brightfield and fluorescent modes at three representative time points for: A) untreated seeds or B) seeds treated with 200 mM NaCl.

Figure 4. Dose-dependent delay of seed germination upon seed treatment with NaCl. Averaged raw data are shown for untreated seeds and seeds treated with increasing concentrations of NaCl. For each condition, the last time point was set at the cycle corresponding to the maximum percentage of seed germination, to avoid biases due to long radicles, as explained in the Results section.
Table 2. Analysis of the percentage of germinated seeds using MyAssays® Desktop. Visualization of the kinetic plots of individual wells, averaged plots and 4PL fitting. Maximum seed germination rate and G₅₀ (the point of inflections), slope at the point of inflection and R² for the fitting are reported. G₅₀ is defined as the time required for the germination of 50% seeds.

<table>
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<th>Sample</th>
<th>Raw Graphs</th>
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<th>4PL fit</th>
<th>Max (%)</th>
<th>G₅₀ (h)</th>
<th>Slope</th>
<th>R²</th>
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Inhibition of Seed Germination by Abscisic Acid

Seeds were seeded in presence of 8 increasing concentrations of ABA (10 nM, 20 nM, 50 nM, 130 nM, 320 nM, 800 nM, 2 µM and 5 µM). The assay was performed in sextuplicate. Control wells contained no ABA. Images were acquired for 24 time points every four hours, for a total time of 92 hours.

Figure 5 shows the brightfield images of seeds acquired after 48 hours of incubation: a high rate of germination is evident in untreated wells (column 11), where relatively long radicles are clearly visible for most seeds. Conversely, wells with increasing concentration of ABA show an evident inhibition of germination; radicles are shorter in wells with intermediate concentration of ABA (columns 6-7-8). Almost no radicles are visible at 2 and 5 µM ABA (columns 3-4).

Figure 5. Wells showing different degrees of seed germination after 48 hours of treatment with ABA. ABA concentration is maximum in column 3 (5 µM) and decreases toward column 10 (10 nM ABA). Column 11 contains untreated, control seeds. For each condition, the assay was performed in 6 wells (vertical replicates).
These qualitative observations were transformed into quantitative data by calculating the percentage of germinated seed and by applying 4PL fitting to averaged data; the results are reported in Table 3.

The maximum percentage of germination of untreated seeds was 95.3% and $G_{50}$ was equal to 21.4 hours, in good agreements with the data shown in Table 2.

Seeds treated with ABA concentrations in the 10-130 nM range reached a percentage of germination $\geq$ 89% and the $G_{50}$ was close to 21.5 hours. Thus, seeds treated with lower concentrations of ABA can reach a maximum germination rate like untreated seeds, with similar speed. Seed treated with 320 nM ABA reached a similar germination percentage, but with a delay ($G_{50}$ equal to 25.9 hours).

Seed germination was hampered by higher concentrations of ABA: the maximum seed germination rates were 80.5% at 800 nM ABA and 48.6% at 2 µM ABA. The highest ABA concentration (5 µM) almost completely inhibited the germination of A. thaliana seeds (data interpolation by 4PL was not applicable for this condition, as highlighted by $R^2$ 0.199 in Table 3 indicating unsuitable data fitting).

Conclusions
We reported a simple and accurate assay based on well imaging to quantify the germination rate of A. thaliana seeds in 96-well plate format. Image acquisition and incubation were automatically performed by the EnSight multimode plate reader. Images were accumulated every four hours for three days and automatically analyzed by the EnSight software, Kaleido.

The time requested for 50% of seeds to germinate in endogenous conditions corresponded to approximately 22 hours. Seeds treatment with NaCl 10-200 mM caused a dose-dependent delay of the time required to reach 50% of maximum seed germination ($G_{50}$) from 23.6 to 81.5 hours; more than 90% of the seeds were able to germinate at all tested NaCl concentrations.

Conversely, seed treatment with the germination inhibitor abscisic acid (10-5000 nM) caused both a delay in germination and a reduced maximum germination rate, in a dose-dependent way. 5000 nM ABA completely inhibited the germination.

Current available methodology to analyze seed germination consists of the visual scoring of seed radicle emergence over several days, making measurements every 24 hours in most cases. The visual approach provides limited information about seed physiological state such as dormancy degree or population uniformity, including tolerance to abiotic and biotic stress. In addition, this experimental set-up limits the number of samples that can be handled in a single experiment. Conversely, the microplate format of the assay based on well imaging allowed testing different experimental conditions in parallel using different replicates to collect statistically significant data. The Kaleido software extracted quantitative data from images immediately following their acquisition, by applying a guided, yet customizable analysis.

The additional software, MyAssays® Desktop, was an ideal tool to average kinetic curves and for data interpolation, to efficiently detect variation of germination rates.

Please send request to your regional PerkinElmer representative for the analysis method of A. thaliana seeds or seeds with similar dimensions and exhibiting endogenous fluorescence in radicles. A variant of the analysis can also be applied to seeds exhibiting localized fluorescence.

Table 3. Inhibition of seed germination by ABA, resulting from analysis using MyAssays® Desktop. Visualization of the kinetic plots of individual wells, averaged plots and 4PL fitting. Corresponding results of data fitting are reported: maximum percentage of germination, inflection point ($G_{50}$), slope at inflection point and $R^2$.

<table>
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<tr>
<th>Sample</th>
<th>Raw Graphs</th>
<th>Average</th>
<th>4PL fit</th>
<th>Max (%)</th>
<th>$G_{50}$ (h)</th>
<th>Slope</th>
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References


Acknowledgements

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