

1 **A bootstrap approach is a superior statistical method for the comparison of cell-to-cell movement**
2 **data**

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8 **Keywords**

9 Plasmodesmata, bootstrap, Mann-Wilcoxon-Whitney, cell-to-cell movement, statistics

10 **Summary**

11 Plasmodesmata are an increasing focus of plant research, and plant physiologists frequently aim to
12 understand the dynamics of intercellular movement and plasmodesmal function. For this,
13 experiments that measure the spread of GFP between cells are commonly performed to indicate
14 whether plasmodesmata are more open or closed in different conditions or in different genotypes.

15 We propose cell-to-cell movement data sets are better analysed by a bootstrap method that tests
16 the null hypothesis that means (or medians) are the same between two conditions, instead of the
17 commonly used Mann-Whitney-Wilcoxon test. We found that that with hypothetical distributions
18 similar to cell-to-cell movement data, the Mann-Whitney-Wilcoxon produces a false positive rate of
19 17% while the bootstrap method maintains a false positive at the set rate of 5% under the same
20 circumstances. Here we present this finding, as well as our rationale, an explanation of the
21 bootstrap method and an R script for easy use. We have further demonstrated its use on published
22 datasets from independent laboratories.

23 **Main Text**

24 Symplastic cell-to-cell connectivity is dynamically regulated in plants as a component of
25 developmental and environmental responses (Perbal *et al.*, 1996; Wada *et al.*, 2002; Faulkner *et al.*,
26 2013). Connectivity is established between cells by plasmodesmata, which function as a key
27 parameter to define the dynamics of cell-to-cell connectivity. It is critical to assay the degree of
28 movement of different molecules between cells to understand the range and dynamics of cell-to-cell
29 communication as well as to assay plasmodesmal function under different conditions or in different
30 genotypes. Accurate experimental analysis is critical to understanding this important component of
31 plant physiology.

32 There are two routinely used methods, with a cellular resolution, to assay the spread of GREEN
33 FLUORESCENT PROTEIN (GFP) from one cell into neighbouring cells: microprojectile bombardment,
34 and low OD₆₀₀ *Agrobacterium tumefaciens* infiltration (Oparka *et al.*, 1999; Burch-Smith & Zambryski,
35 2010). These assays allow the experimenter to count the number of cells ('cell count'), or the
36 number of concentric rings of cells ('cell layers'), to which GFP has spread from a single cell (Fig. **1a**).
37 This serves as a measure of symplastic connectivity – the further the GFP has spread, the greater the
38 degree of connection (or of plasmodesmata permeability) between cells. Neither cell nor layer
39 counts are parametrically distributed (Fig. **1b – d**, upper), so most studies use the non-parametric
40 Mann–Whitney–Wilcoxon (MWW) test to compare conditions to identify factors that regulate the
41 connection and communication between cells.

42 Most experiments aim to assess whether connectivity is greater or less under different conditions, or
43 whether plasmodesmata are more open or closed, which involves analysis of changes in the median
44 or mean. The MWW tests the null hypothesis that two data distributions are the same (Mann &
45 Whitney, 1947), not whether the two distributions have the same median. Therefore, it is possible to
46 find a significant difference in an MWW test with distributions that have the same median, but
47 different variances (Hart, 2001). When data from cell count assays are presented in histogram form,
48 it is clear that the shape of the distributions differs between experimentally compared conditions or
49 genotypes (Fig. **1b, c**) (Guseman *et al.*, 2010; Diao *et al.*, 2018; Cheval *et al.*, 2020). Thus, if an MWW
50 test is used on cell count data, the difference in distribution shapes between conditions may lead to
51 the erroneous conclusion that there is a significant difference in the amount of spread of GFP.

52 Therefore, a different statistical method is required to properly interpret differences in GFP spread.
53 For this, we propose a bootstrap method (Efron, 1979). Unlike the MWW test, bootstrapping works
54 with data that is both non-parametric and heteroskedastic (differing variance between conditions).

55 The goal of the analysis is to estimate the probability that the observed difference in medians ($\hat{\theta}$)
56 came about by chance (a *p* value). Frequentist statistics does this by comparing $\hat{\theta}$ to a null
57 distribution. In this case, the null distribution describes the probability of observing a difference in
58 medians, when there is no true difference in the underlying data. Usually, a known distribution is
59 used (e.g. t-distribution or F-distribution) but in this case it is unknown because the data do not
60 follow parametric distributions (Fig. **1b, c**).

61 Bootstrapping techniques can be used to generate a null distribution *de novo* from the observed
62 data already collected, as long as the samples are independent. This removes the requirement of
63 using a known distribution. To do this, the observed data are sampled with replacement to generate
64 a resample. This mimics what the experimenter has done originally when observing the true

65 population. The relationship between multiple resamples and the observed data can be used to
66 reveal how the observed data relate to the true population, and so estimate a p value for the
67 observation.

68 An example R function is provided to perform this analysis (*medianBootstrap.R*,
69 <https://github.com/faulknerfalcons/Johnston-2020-Bootstrap>), which requires two arguments, i.e.
70 two vectors of numbers: control and treatment. The function generates a null distribution to
71 compare against by resampling each vector N times (by default 5000) and, for each resample,
72 generating a resampled test statistic ($\widehat{\theta}^*$). These N resampled test statistics are made into a null
73 distribution by $|\widehat{\theta}^*_n - \widehat{\theta}|$ (Fig. **1b – c**, lower) as suggested by Hall and Wilson (1991).

74 As this is a random sampling technique, an exact p value cannot be calculated but an estimate is
75 produced: a Monte Carlo \hat{p} value (Eqn. 1). To do so, $\widehat{\theta}$ is compared to the null distribution to find the
76 chance of observing a value at least as extreme (line on Fig. **1b – c**, lower). A +1 is added to the
77 numerator and denominator in Eqn. 1 as suggested by Davison and Hinkley (1997): conceptually, this
78 can be considered as including the observed sample among the bootstrap resamples.

$$\hat{p} = \frac{\sum_{n=1}^N I(|\widehat{\theta}^*_n - \widehat{\theta}| \geq \widehat{\theta}) + 1}{N + 1} \quad \text{Eqn. 1}$$

79 where $I(\cdot)$ is the indicator function.

80 As \hat{p} is an estimate of p , a 95% confidence interval should be constructed, where p will fall within
81 this range 95% of the time (Wilson, 1927).

82 This method is not confounded by differences in variance or shape as with the MWW test. To
83 illustrate this, we compared the Type I error rate (false positives) between the MWW and
84 *medianBootstrap* tests, when testing if there is a difference in medians between two populations for
85 which there was no true difference in medians, i.e. $\theta = 0$. In this scenario, an error rate of 5% is
86 expected at $\alpha = 0.05$. Samples ($n = 100$) for each population were drawn from normal
87 distributions with the same variance ($X, Y \sim N(0,1)$) simulated in R 4.0.0 (R Core Team, 2020). Both
88 the MWW and *medianBootstrap* methods gave a difference in medians about 5% of the time, as
89 expected (4.5% (95% CI [3.4, 6.0]) and 4.9% (95% CI [3.7, 6.4]), respectively). When variances
90 differed between populations ($X \sim N(0,1), Y \sim N(0, 5^2)$), the MWW test had a false positive rate
91 significantly higher than the set 5% of 7.5% (95% CI [6.0, 9.3]). Conversely, the false positive rate of
92 the *medianBootstrap* method was correctly controlled at 4.7% (95% CI [3.6, 6.2]).

93 Alternatively, when two samples are drawn from populations with equal variance and median, but
94 differing shape ($X \sim N(1 - \frac{1}{\sqrt{2}}, \frac{3}{80}), Y \sim \text{Beta}(1,3)$), a *medianBootstrap* method finds a significant

95 difference in 5.1% of the trials (95% CI [3.9, 6.6]), as expected. Whereas, an MWW test inflates the
96 Type I error rate to 17% (95% CI [15, 19]). Therefore, as cell count data exhibit unequal variances and
97 differing distribution shapes between conditions and/or genotypes, we propose that bootstrap
98 methods are a more appropriate analysis to identify differences in the spread of GFP. It is worth
99 noting that any test statistic, θ , can be computed in a bootstrapped manner, provided the test is
100 invariant to scaling. This means bootstrap testing can be extended to cell layer data, where means
101 should be compared, as there is no difference in medians (Fig. 1d). An example of this extension is
102 given in *medianBootstrap.html*.

103 We acknowledge alternative advanced statistical techniques, such as linear mixed effects models, for
104 the analysis of these data. However, they require more assumptions and are less user-friendly. We
105 consider this bootstrap method a good, easy-to-use, superior alternative to MWW analysis of cell-to-
106 cell movement data.

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116 Author Contributions

117 MGJ and CF designed, discussed and wrote up the research. MGJ performed the analysis.

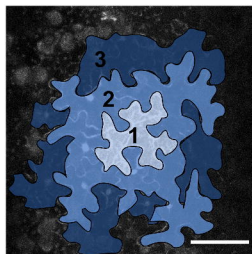
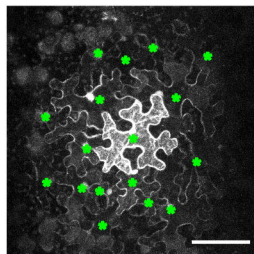
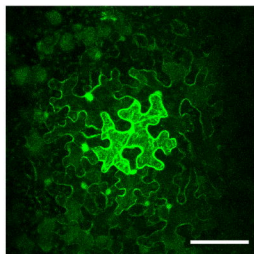
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- 152 **Figure 1 Bootstrap statistics on GFP movement data.**

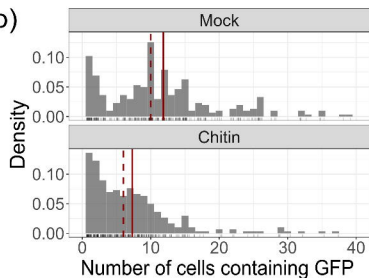
153 **(a)** An example image of GFP moving from a single transformation site. The degree of movement can
154 either be counted as the number of fluorescent cells (denoted with stars, 17 cells) or the number of
155 cell layers with GFP (blue overlays, 3 layers). Scale bar = 100 μm . **(b – d) Top:** Histogram of cell
156 counts or layers, with the median and mean marked. **Bottom:** Bootstrap null distributions ($|\hat{\theta}^* - \hat{\theta}|$)
157 for the differences in **(b, c)** median or **(d)** mean, with estimated \hat{p} value and 95% confidence intervals
158 (CI). The observed difference ($\hat{\theta}$) is marked by a red line. Data for **(b)** from Cheval *et al.* (2020) and
159 data for **(c, d)** from Diao *et al.* (2018) both under the CC BY 4.0 licence.

a)

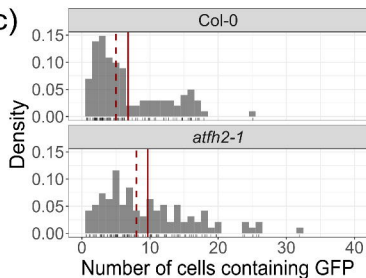


- Median
- Mean
- θ Test statistic
- $\hat{\theta}$ Sampled test statistic
- $\hat{\theta}^*$ Resampled test statistic
- Δ_μ Difference in means
- Δ_{median} Difference in medians

b)



c)



d)

