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Technical note

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3 **Quantification of Pollen Tube Attraction in Response to Guidance by**
4 **Female Gametophyte Tissue Using Artificial Microscale Pathway**

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Abstract

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29 We developed two types of artificial platforms, T-junction and crossroad microchannel devices,
30 and obtained guidance response ratio of pollen tubes to the female tissue as 56–57%. The
31 crossroad device was also able to collect the attracted pollen tubes with high purity, which is
32 useful for future omics analysis.

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39 **Keywords:** Plant reproduction, Pollen tube, Ovule, Pollen tube guidance, *Torenia fournieri*,
40 purification, PDMS, Microchip, Microchannel, MEMS

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51 Pollen tube guidance plays an essential role in plant reproduction because fertilization and
52 subsequent seed development cannot be achieved without pollen tubes (PTs) delivering two
53 sperm cells to an egg cell and to a central cell in the female gametophyte, respectively. While
54 signals from female gametophyte tissue are believed to guide the PTs to the embryo sac,
55 interaction between the PT and female gametophyte tissue is essential for successful fertilization
56 in plants. Therefore, precise quantification by means of a PT guidance assay is an important
57 objective to gain an improved understanding of the mechanism of PT guidance. An *in vitro*
58 guidance assay has been performed on an agarose medium with ovules of *Torenia fournieri* and
59 the guidance ratio has been defined using the total number of ovules as the population [1-3].
60 However, not all germinated PTs show capability for the guidance response to an attractant
61 released from ovules and at least germination of pollen grains on the stigma and elongation of
62 PTs through the cut style are essential for acquisition of the guidance response capacity [1].
63 Therefore, measurement of the guidance response ratio (GRR) of PTs, defined as the proportion
64 of guided PTs in the total population of PTs, is important for exploration of the mechanism of
65 PT guidance. In the previous studies described above, however, the GRR of PTs was not
66 measured probably because it was difficult to determine whether or not PTs were guided during
67 variable growth of PTs under the bulk condition on an agarose medium in a petri dish.

68

69 To allow access to measure the GRR of PTs, miniaturized microdevices, represented by a
70 micro-electro-mechanical systems (MEMS), show various advantages in functionality compared
71 with their macroscopic counterparts with decreasing scale [4]. In general, the volume effect is
72 reduced more than the surface effect in down-scaling. Taking advantage of the physical
73 phenomena of a microscale platform, various novel types of biological experiments have been
74 realized using microdevices [5], such as a microheater [6], microchamber array [7], and
75 microfluidic devices [8], frequently in combination with micro/nano manipulation techniques

76 [9]. Microchannel devices or microfluidic systems have been adopted, in the past few years, to
77 perform PT growth assays efficiently and quantitatively. The on-chip method has considerable
78 potential as a future platform for studies of plant reproduction because it is expected to allow
79 not only precise handling of PTs but also to create and control the chemical environment at
80 micrometer-scale resolution. Such technology can be used to develop a microenvironment
81 mimicking that within the pistil during *in vivo* PT growth. Given these advantages, PT growth
82 rate measurement has become possible under conditions that closely simulate the *in planta*
83 environment [10]. A microsystem-based assay has been successfully adopted to develop a
84 microfluidic network to examine the cellular penetrative forces [11] and the device was further
85 modified for experimentation and phenotyping of PTs [12]. An on-chip guidance assay was first
86 performed in a relatively “large” microchannel that held a pistil of *Arabidopsis thaliana* and
87 allowed the PTs to grow in the channel filled with a gel-based pollen growth medium [13]. An
88 advanced on-chip guidance assay was realized by adopting *Torenia fournieri*, whose pollen
89 tubes grow faster and longer than those of *A. thaliana*. Consequently, PT growth orientation at
90 the T-junction showed “left or right” distinction, which made the guidance assay more efficient
91 for further screening of materials that may be involved in PT attraction [14]. However, in these
92 assays, the GRR is unavoidably overestimated because, in addition to the attracted PTs, whether
93 non-attracted PTs grow towards or away from the female gametophyte tissues is assumed to be
94 of equal probability.

95

96 In this report, we propose a method to measure the net GRR of PTs quantitatively using
97 microchannel devices of simple design, namely the “crossroad device” and the “T-junction
98 device”. Using this method, we report that the net GRR of PTs was measured to be 56–57%
99 under the experimental condition. In addition, we achieved enrichment of attracted PTs in the
100 ovule-containing microchannel reservoir at the percentage of 87% using the crossroad device.

101

102 Two types of polydimethylsiloxane (PDMS) microchannel devices were prepared in this study.
103 Both the T-junction and crossroad devices consist of a buffer and style inlet, buffer outlets, and
104 microchannels of 500 μm in width and 25 μm in height. Thus, the device allows for growth
105 assays of multiple PTs passing through the same pistil (Fig. 1). In the T-junction device, the
106 distance between the center of the style inlet and the T-junction was 3.5 mm. In the crossroad
107 device, the distance between the center of the style inlet and the crossroad was 2 mm. The
108 microchannel devices were developed using the fabrication process described elsewhere [15].
109 Briefly, the negative master for the molding of PDMS was fabricated on a silicon wafer with an
110 ultra-thick photoresist (SU-8 3025; Microchem Corp., Newton, MA, USA). The prepolymer of
111 PDMS (Sylgard® 184, Dow Corning, Midland, MI, USA) was cast onto the master, which had
112 a frame to hold the prepolymer in place. The prepolymer was degassed in the vacuum chamber
113 for more than 20 min. After incubation at 65 °C for 90 min, the cured PDMS was peeled off
114 from the master, and the through-holes for the style (1.2 mm in diameter) and buffer inlets (3
115 mm in diameter) were punched out manually.

116

117 The experimental protocols were as follows. The PDMS microchannel device was placed in the
118 middle of a glass-bottomed dish. After degassing the device in a vacuum chamber at 10 kPa for
119 at least 40 min, 20–40 μL modified Nitsch's medium [16] was injected from the style inlet to fill
120 all of the channels via the power-free pumping mechanism of the PDMS microchannel device
121 [17,18]. A hand-pollinated style of *Torenia fournieri* cv. blue and white, cut to a length of 1 cm,
122 was placed in the style inlet of the device. A placenta possessing approximately 500 ovules from
123 a mature *T. fournieri* flower was placed in the buffer inlet. Concentration of attractant at the
124 junction of the crossroad device can be lower than at the junction of the T-junction device.
125 However, since we confirmed that 10~20 ovules in buffer inlet can induce PTs in the T-junction

126 device (Kuzuya, et al., unpublished data), a placenta possessing 500 ovules create sufficient
 127 concentration of attractant also at the junction of the crossroad device. After pollination, the
 128 pollen tubes grew through the style and entered the channels. The dish was kept moist in an
 129 incubation chamber at $25 \pm 1^\circ\text{C}$. The measurement of the GRR of PTs was assessed 16~20 h
 130 after pollination. For microscopic observation of PTs in the microdevice, we used a
 131 stereomicroscope (Axio Zoom V16; Zeiss, Germany, MVX-10; Olympus, Japan) equipped with
 132 a CCD camera (AxioCam MRC; Zeiss ,DP72; Olympus). For image processing, image analysis
 133 software, ImageJ, was used (<http://imagej.nih.gov/ij/>).

134

135 The GRR of PTs can be calculated from the number of PTs entering a ovule-containing
 136 microchannel (Fig. 1). In the T-junction device, given that non-attracted PTs growing towards or
 137 away from the female tissues are assumed to be of equal probability, the proportion of PTs
 138 entering the microchannel directed towards the ovules over the total number of PTs (RTt) is
 139 expected to be

$$140 \quad RTt = GRR + \frac{100 - GRR}{2}, \quad (1)$$

141 where GRR represents the net guidance response ratio. This equation is based on the premise
 142 that hemorepulsion response between PTs and between PTs and ovules do not take place during
 143 our guidance assays, because the repelling response has not been observed in *in-vitro* assays
 144 until PTs get close to ovules within short range at a distance $\sim 100 \mu\text{m}$ [19]. The ratio of PTs
 145 entering the microchannel directed away from the ovules over the total number of PTs (PTa) is
 146 expected to be

$$147 \quad PTa = \frac{100 - GRR}{2}. \quad (2)$$

148 The measured frequencies of PTs growing towards (PTt) and away from the ovules (PTa) by

149 repeated experiments were $78.1 \pm 6.5\%$ and $21.9 \pm 6.5\%$ (mean $\pm$ S.D., $n = 16$), respectively (Fig.
150 2A). In the absence of ovules the respective values were $50.3 \pm 9.8\%$ and $49.7 \pm 9.8\%$ ($n = 11$)
151 (Fig. 2B). Consequently, PTs were significantly attracted towards the ovules ($p < 0.01$, Student's
152 t -test) and the net GRR was calculated to be 56.2% for the assay using the T-junction device. On
153 the other hand, for the crossroad device the ratio of PTs entering the microchannel towards the
154 ovules over the total number of PTs (R_{ca}) is expected to be

$$155 R_{ca} = GRR + E, \quad (3)$$

156 where E (error) represents the proportion of PTs entering channels both towards and away from
157 ovules by chance without any guidance effect. The ratio of PTs entering the microchannel away
158 from the ovules over the total number of PTs is expected to be equal E, which we assumed to be
159 the same value as in equation (3). The measured values for PTs growing towards (R_{ca}) and
160 away from the ovules (E) were $65.1 \pm 17.8\%$ and $8.2 \pm 3.5\%$ ($n = 56$), respectively (Fig. 2C);
161 the remainder of the PTs grew straight through the crossroad. In the absence of ovules, E values
162 for right and left channel showed no significant difference as expected (Fig. 2D). Consequently,
163 the GRR was calculated to be 56.9% for the assay using the crossroad device.

164

165 The GRR measured with the two independent designs was extremely consistent. Therefore, the
166 GRR under the present *in vitro* condition was determined to be 56–57% with high reliability.
167 Additionally, the crossroad device was advantageous for collection of the attracted PTs with
168 higher purity because the percentage of attracted PTs with the crossroad device was 87.4%
169 ($56.9/65.1$) and 56.2% with the T-junction device.

170

171 In summary, we measured and quantified the net GRR of PTs by developing an artificial
172 microscale assay platform, namely two types of microchannel devices of simple design: the
173 T-junction device and the crossroad device. The GRRs measured with both devices were highly

174 consistent and under the present experimental condition the GRR was determined to be 56–57%
175 with extremely high reliability. Furthermore, the crossroad device enriched the attracted PTs.
176 The homogenous sampling paves the way for collection of precise data for transcriptome and
177 proteome analysis of the attracted pollen tubes. We recently developed MEMS-based PDMS
178 microcage arrays to position and fix ovules for long-term live imaging [20]. Combining this
179 microcage array to handle an ovule, female tissue, and the PT guidance assay proposed in the
180 present report might realize a more complex and sophisticated assay system in the future.

181

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246

247 **Figure Captions**

248 **FIG. 1.** Photographs of the T-junction device (A) and the crossroad device (B)
249 for measurement pollen tube guidance response ratio. Attracted pollen tubes
250 by guidance signal from ovules are illustrated in red.

251

252 **FIG. 2.** Representative microscopic images of pollen tubes growing in the T-junction device (A,
253 B) and the crossroad device (C, D). Pollen tubes were attracted in both channels in the presence
254 of ovules (A, C), but not in the absence of ovules (B, D).

Figure 1

Figure 2