

Supplementary Information

A universal platform for selection and high-resolution phenotypic screening of bacterial mutants using the nanowell slide

H. Antypas^a, M. Veses-Garcia^a, E. Weibull^{b,#}, H. Andersson-Svahn^b,
A. Richter-Dahlfors^{a,*}

Affiliations

^a Swedish Medical Nanoscience Center, Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden.

^b Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, KTH-Royal Institute of Technology, Stockholm, Sweden.

[#] Current address: Vironova AB, Gävlegatan 22, 113 30 Stockholm

* Correspondence should be addressed to: agneta.richter.dahlfors@ki.se

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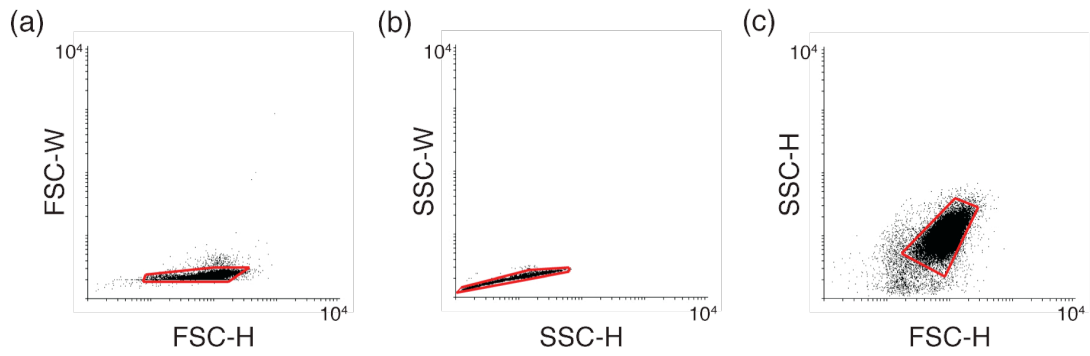


Figure S1. Gating strategy for sorting of single bacteria.

(a) FSC-W vs FSC-H plot, and (b) SSC-W vs SSC-H plot were used to select for *E. coli* W3110 wt loaded in the FACS instrument.

(c) SSC-H vs FSC-H plot was used to further interrogate bacteria from (a) and (b), and gate for single bacteria.

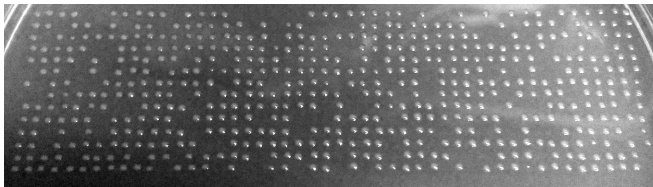


Figure S2. Colonies originating from individual bacteria sorted in a nanowell pattern on LB agar plates.

The alignment of the FACS motorized stage with the grid of the nanowell slide is visualized by the sorting of single bacteria onto LB agar plates. Following overnight incubation, an organized pattern of colonies appears, where each colony corresponds to the location of each nanowell on the nanowell slide.

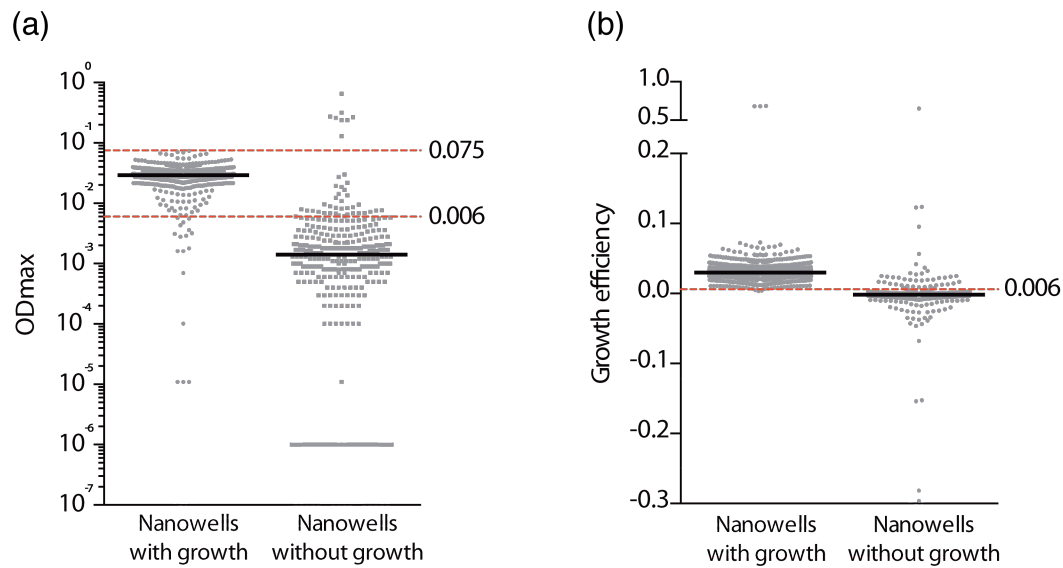


Figure S3. Threshold determination of nOSAT checkpoints

(a) OD_{max} values from 977 nanowells from three independent experiments after overnight incubation at 37°C. Microscopic examination revealed 644 nanowells as true positive for growth (Nanowells with growth) and 353 nanowells as negative for growth (Nanowells without growth). Median OD_{max} in each group is shown by a black line. The range $0.006 < OD_{max} < 0.075$ defines the upper and lower threshold (red lines) that differentiates nanowells with true growth from artifacts.

(b) The growth efficiency in all nanowells from (a), with the median growth efficiency in each group shown by a black line. A value ≥ 0.006 is defined as the growth efficiency threshold (red line) able to discriminate between nanowells with and without growth.

Table S1. Comparison of the nwSlide with a standard 384-well plate

	Technical specifications	
	Standard 384-well plate	nwSlide
Number of wells	384	672
Plate length x width	127.76 x 85.48 mm	75 x 25 mm
Plate height	7.5 - 14.4 mm	0.675 mm
Well height	5.5 - 12.4 mm	0.5 mm
Well surface	2.66 - 10.89 mm ²	0.4225 mm ²
Well spacing (center to center)	4.5 mm	1.5 mm
Well volume	4-145 μ l	0.5 μ l
Compatibility for microbiology		
	Standard 384-well plate	nwSlide
Bacterial inoculation		
Manual Pipetting	Yes	Yes
Automatic pipetting	Yes	Yes
Single-cell sorting	Yes	Yes
Smearing	No	Yes
Microfluidics-based loading	No	Yes
Spectrophotometric monitoring of bacterial growth	+	++
Microscopic analysis of bacteria	+	++
Retrieval of bacteria	Yes	Yes
Reagent consumption per plate	1.5 - 55.7 ml	0.34 ml
Reusability	No	Yes (autoclavable)

Table S2. Primers and plasmids used in this study

Primer	Sequence (5'-3')	Reference
Kan-FP	CCATGGCGATGCCTGCTTGC	This work
fliT-RP	CATAGGAGTCGTAGCCGACCGA	This work
Sq-FP	GCCAACGACTACGCACTAGCCAAC	Epicentre, USA
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG	Epicentre, USA
T6D4_FW	TCAGTTGCTGTTGACTGTA	This work
T6D4_RV	CACAAGGTAAACAGTTGAT	This work
RATE-FP	GATTACACATGGCATGGA	This work
RATE-FP2	TACAAATAAATGTCCAGACC	This work
RATE-FP3	CCTGCAGGCATGCAAGCTT	This work
Inv-1	ATGGCTCATAACCCCCTTGTATTA	Epicentre, USA
SacI-Kan-FP	ATAGAGCTCGTGTAGGCTGGAGCTGCTTC	This work
KpnI-Kan-RP	TCAGGTACCATGGGAATTAGCCATGGTCC	This work
Sall-GFP-FP	AGCAGTCGACGTAAAACGACGGCCAGT	This work
HindIII-GFP-RP	CCGCCAAAACAGCCAAGCTT	This work
ME Plus 9-3'	CTGTCTCTTATACACATCTCAACCATCA	Epicentre, USA
ME Plus 9-5'	CTGTCTCTTATACACATCTCAACCCTGA	Epicentre, USA
Plasmid		Reference
pMOD-2	ampR, MCS	Epicentre, USA
pMOD-2-KAN	ampR, kanR	This work
pMHA	ampR, kanR, <i>gfpmut2</i>	This work
pKD4	ampR, kanR	¹
pKEN GFPmut2	ampR	²

Table S3. PCR cycling conditions

Primer pair	Cycles (No)	PCR conditions		
		Denaturing	Annealing	Extension
Kan-FP & flIT-RP	26	94°C, 45 s	68°C, 60 s	72°C, 150 s
T6D4_FW & T6D4_RV	30	94°C, 30 s	55°C, 30 s	72°C, 180 s
SacI-Kan-FP & KpnI-Kan-RP	30	98°C, 10 s	55°C, 30 s	72°C, 60 s
Sall-GFP-FP & HindIII-GFP-RP	30	98°C, 10 s	59°C, 30 s	72°C, 60 s
ME Plus 9-3' & ME Plus 9-5'	30	98°C, 10 s	55°C, 30 s	72°C, 90 s
RATE-FP ¹	30	94°C, 45 s	52°C, 30 s	72°C, 30 s
	30	94°C, 45 s	30°C, 30 s	72°C, 30 s
	30	94°C, 45 s	52°C, 30 s	72°C, 120 s
RATE-FP2 ¹	30	94°C, 45 s	49°C, 30 s	72°C, 30 s
	30	94°C, 45 s	30°C, 30 s	72°C, 30 s
	30	94°C, 45 s	49°C, 30 s	72°C, 120 s
RATE-FP3 ¹	30	94°C, 45 s	52°C, 30 s	72°C, 30 s
	30	94°C, 45 s	30°C, 30 s	72°C, 30 s
	30	94°C, 45 s	52°C, 30 s	72°C, 120 s
Inv-1 ¹	30	94°C, 45 s	55°C, 30 s	72°C, 30 s
	30	94°C, 45 s	30°C, 30 s	72°C, 30 s
	30	94°C, 45 s	55°C, 30 s	72°C, 120 s

¹Single-primer PCR

Supplementary Note 1

Advantages of the nwSlide over the 384-well plate for use in microbiology

Bacterial inoculation

Inoculation of bacteria on the nwSlide can be performed in 5 different ways (Table S1), depending on the researcher's needs. In the current work inoculation by smearing and single-cell sorting was used. Contrary to the 384-well plate, which has a plate height of more than 7.5 mm, the 0.675 mm thickness of the nwSlide enables smearing of a bacterial sample across all nanowells³. This is performed with a single pipetting sample deposition on the nwSlide and smearing across nanowells using a membrane. The total time for this procedure is less than a minute. The nwSlide is also compatible with FACS. The well spacing distance (center to center) of the nwSlide is designed to match the movement of the plate holder in FACS instruments, which enabled sorting of single mammalian cells⁴, and single bacteria in the current work within 15 minutes. Although not yet explored, this well spacing distance is also compatible with pipetting robots to enable automatic liquid handling. Finally, the nwSlide can be integrated with a microfluidic component to facilitate sample loading and medium exchange in the nanowells⁵, a feature unavailable to standard 384-well plates.

Spectrophotometric monitoring of bacterial growth

The nwSlide and the 384-well plate are both compatible with spectrophotometry to monitor bacterial growth (Table S1). Owing to a 3D-printed custom adaptor, the nwSlide becomes compatible with a conventional plate reader. When it comes to monitoring growth in wells inoculated with single bacteria, the nwSlide is better suited due to the ≥ 8 times smaller volume capacity of the nanowells. Bacteria should reach a concentration of $\geq 10^7$ cfu/ml for growth to be detectable by absorbance recordings⁶. Therefore, a culture starting from a single bacterium reaches detectable levels in the 0.5 μ l of a nanowell faster compared to the ≥ 4 μ l of a 384-well plate.

Microscopic analysis of bacteria

Having the dimensions of a microscopy slide (75 x 25 mm) and a glass bottom of 175 μ m, the nwSlide is ideal for microscopy. Although variations of the 384-well plate with a glass bottom exist, they are far from optimal for imaging of bacteria. Their relatively large well height and surface area (Table S1) are not suitable to study growth of single motile bacteria. In contrast, the significantly smaller dimensions of the nanowells facilitate detection and monitoring of single bacteria.

User-friendliness of the nwSlide

The nwSlide requires a total reagent volume of ≈ 336 μ l, which is at least 4.5 times less compared to the 384-well plate (Table S1). The low volume can help reduce laboratory costs, but also disposal of reagents in the environment. Moreover, the low volume requirement is advantageous for clinical sample testing. Another significant advantage over the 384-well plate is that the nwSlide is autoclavable, and can therefore be reused several times. This further reduces costs and waste.

Supplementary Note 2

The nanoculture Optical Signal Analysis Tool (nOSAT) algorithm

To discriminate between nanowells with and without bacterial growth we developed nOSAT. This algorithm uses the absorbance recordings of each nanoculture in the nwSlide to control whether the nanocultures pass the following checkpoints:

Checkpoint 1. Does the nanoculture transition from lag to logarithmic phase thereby generating a T_{lag} ?

Checkpoint 2. Does the OD_{max} of the nanoculture fall between 0.006 - 0.075?

Checkpoint 3. Is the growth efficiency ≥ 0.006 ?

Before applying the nOSAT algorithm, the baseline absorbance was subtracted from each nanowell. As baseline we defined the minimum absorbance recorded in the first 8 time points. All subsequent nOSAT calculations were performed in Excel (v14.6.4, Microsoft).

Checkpoint 1: T_{lag} determination

To check whether a culture transitions from lag to logarithmic phase, we calculated T_{lag} . For a full description of this previously developed algorithm, readers are referred to reference 3. Briefly, the T_{lag} algorithm is composed of the following:

1) Calculation of the change in average optical density over time (ΔMOD):

$$\Delta MOD = \frac{\sum_{i+2}^i OD}{3} - \frac{\sum_{i-2}^{i-1} OD}{3}$$

2) Calculation of the difference between sequential ΔMOD values ($\Delta \Delta MOD$):

$$\Delta \Delta MOD = \Delta MOD_{t_{n+1}} - \Delta MOD_{t_n}$$

3) A control step to check if sequential $\Delta \Delta MOD$ values exceed a certain threshold:

If $\Delta \Delta MOD \geq 0.00038$ for 3 subsequent time points ($t_n \dots t_{n+3}$), then $t_n = T_{lag}$

The above formulas of the T_{lag} algorithm were adjusted to a 30 min interval between absorbance recordings by calculating ΔMOD from 5 time points and setting the $\Delta \Delta MOD$ threshold to 0.00038.

To evaluate the performance of the algorithm, we applied it on data sets of absorbance recordings from three independent experiments on the nwSlide. These were obtained after sorting single bacteria on the nwSlide, incubating them at 37°C and monitoring OD_{600} every 30 min for 16 h. First, we applied the T_{lag} algorithm on these recordings to mathematically determine whether nanowells had bacterial growth or not. We then randomly selected a representative number of nanowells from each slide and used microscopy to inspect whether the nanocultures were positive or negative for bacterial growth. By comparing microscopy results with the T_{lag} results, we made the following definitions:

- True positives (TP): Nanowells confirmed with bacterial growth both microscopically and with the T_{lag} .
- True negatives (TN): Nanowells confirmed without bacterial growth both microscopically and with the T_{lag} .

- False negatives (FN): Nanowells confirmed with bacterial growth microscopically but not with the T_{lag} .
- False positives (FP): Nanowells confirmed without bacterial growth microscopically but were identified with bacterial growth with the T_{lag} .
- Sensitivity was calculated by $(TP/(TP+FN))$, resulting in 97.24 %.
- Specificity was calculated by $(TN/(TN+FP))$, resulting in 75.97 %.

Checkpoint 2: OD_{max} of nanocultures

Specificity for the T_{lag} algorithm showed that there is a number of nanowells without growth, misidentified as having growth (false positives). These empty nanowells would still generate a T_{lag} due to sudden absorbance fluctuations over time. Some of these false positives nanowells, however, would either remain within a relatively low range of absorbance units or they would reach abnormally high absorbance values. We therefore calculated the OD_{max} for each nanowell, and plotted these values separately for nanowells with and without growth to analyze their distribution. Only a small overlap between nanowells with and without growth was observed for the OD_{max} distribution (Fig. S3a). Taking into account the small overlap of the 2 distributions, we set the lower threshold for expected OD_{max} from bacterial nanocultures at 0.006 and the upper threshold for expected OD_{max} at 0.075. When we filtered the data with both this condition and the T_{lag} algorithm, we achieved 95.63 % sensitivity and 90.99 % specificity.

Checkpoint 3: Growth efficiency of nanocultures

We then checked some of the remaining false positive nanowells, and observed that absorbance would suddenly increase and then drop again, reaching the initial absorbance levels. The presence of these peaks would generate a T_{lag} and would also give an OD_{max} within the 0.006-0.075 range, misidentifying these cultures as positive. To exclude these nanowells, we included a 3rd condition in the algorithm so that growth efficiency ($OD_{endpoint} - OD_{baseline}$) must exceed a certain value. We calculated growth efficiency for each nanowell and plotted these values according to nanowells with and without true bacterial growth (Fig. S2b). We observed that wells without bacterial growth had lower growth efficiency compared to nanowells with growth. We set a threshold at 0.006 for growth efficiency, which included the majority of the positive nanowells and only some of the negative nanowells. Combination of this condition with the T_{lag} and OD_{max} , achieves 95.24 % sensitivity and 98.77 % specificity.

By controlling a nanoculture for all the above 3 conditions, we can accurately determine whether it is positive or negative for bacterial growth.

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