Identification and growth rate quantitation of individual bacterial clones using a novel microfluidic concentration device

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Introduction

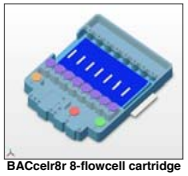
A broad body of medical evidence shows that mortality is significantly decreased when appropriate antimicrobial therapy is initiated within a few hours of a patient presenting symptoms of severe pneumonia. While the physician must decide on therapy within hours of symptom onset, the gold standard diagnostic tool remains microbial culture, a technique that requires days to complete and is biased toward fast growing, majority organisms in a sample.

As a result, empiric therapy is initiated long before the infectious agent is identified. New diagnostic tools that provide microbial identification and antibiotic susceptibility testing within 8 hours of specimen collection will help guide appropriate initial treatment of pneumonia, yielding better outcomes, avoiding unnecessary antibiotic exposure, and reducing emergence of resistance.

The Baccelr8r™ is a new device that allows immunochemical microscopic identification and quantitative growth rate measurements by evaluating in near real time and in situ the mass increase of individual microorganisms. The technique provides a rapid, accurate evaluation of growth dynamics for the population of viable organisms in the sample. This represents a critical component of a rapid, integrated antibiotic susceptibility testing instrument.

Materials and Methods

The experimental system is a bench top instrument that combines a disposable fluidic cartridge with automated microscopy and image analysis software.



BACcelr8r 8-flowcell cartridge

Instrument Features:

- Automated sample distribution to multiple on-board analysis chambers providing integrated electrokinetic concentration and imaging
  - Electrophoretic concentration to a capture & imaging surface using transparent indium tin oxide (ITO) electrodes and redox buffer system
  - Phase contrast, darkfield, and fluorescence microscopy
- XYZ motion control including autofocus
- Off-board (instrument-based) pumps and fluid media
- On-board reagent reservoirs (antibodies, stains, antibiotics)
- Active on-device valving for fluidic network control

Assay Concept

- Off-board specimen prep (simple centrifugation protocol)
- Rapid concentration of bacteria to assay capture and imaging surface using electrokinetic concentration
- Bacterial identification by fluidic introduction of species specific antibodies followed by fluorescently labeled secondary antibodies, with automated epi-fluorescent microscopy
- Individual clone mapping and growth rate quantitation exploits registered time-lapse image analysis, processed to derive growth rate information (doubling times and growth rate constants).
- On-board, near real-time antibiotic susceptibility testing (AST)

Results 1: Electrokinetic Concentration and Capture

A flowcell is built using Indium Tin Oxide (ITO; conductive and transparent) coated glass as top and bottom layers, with adsorptive chemical coating on the bottom surface. A sample containing bacteria is introduced and potential is applied. Since bacteria are generally negatively charged, they migrate to the positively charged surface, where they adsorb to the chemical coating.

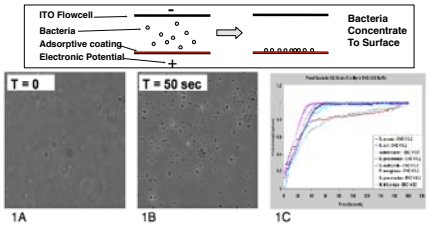


Figure 1. Electrokinetic concentration (EKC) of *S. aureus*. Phase microscopy image at Time = 0 (1A) shows out-of-focus microbes in bulk solution beyond the image plane (surface). Image field of view is 202 x 187 um. After 50 seconds of EKC, microbes have been driven to the surface and concentration is complete (1B). During EKC, phase images are collected at a rate of approximately one per second, and image analysis software is used to rapidly count particles (microbes). Figure 1C shows EKC curves for a panel of 8 clinically relevant bacterial species.

Very rapid concentration and adsorption of bacteria to flowcell surface allows microscopic analysis

Results 2: Bacteria identification with immunolabeling

***Bacterial species display unique surface antigens enabling specific* immunolabeling. Proof of concept was demonstrated by concentrating a sample of mixed Klebsiella pneumoniae and Haemophilus influenzae to a surface and incubating with a mixture of anti-Kp and anti-Hi. Subsequent species-specific secondary fluorescent labeling identified bacterial species via fluorescent imaging.**

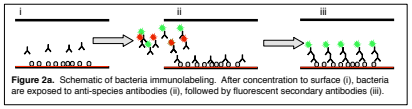


Figure 2a. Schematic of bacteria immunolabeling. After concentration to surface (i), bacteria are exposed to anti-species antibodies (ii), followed by fluorescent secondary antibodies (iii).

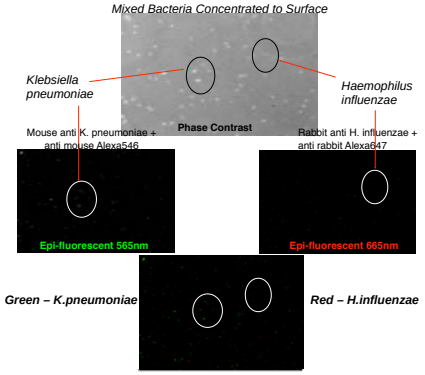


Figure 2b. Immunolabeling of selected bacterial strains. A mixed sample of *Klebsiella pneumoniae* ATCC 49472 and *Haemophilus influenzae* ATCC 10211 was concentrated in a flowcell. A mixture of anti- *K.pneumoniae* and anti- *H.influenzae* was incubated in the cell, followed by fluorescently labeled secondary antibodies. Image acquisition with fluorescent microscopy demonstrates bacterial species labeling.

Identify bacterial species by multiplexed antibody labeling

Results 3: Organism viability within the device

After electrokinetic concentration, the device is automatically filled with growth media (TSB). All subsequent assay steps are performed in media and microorganism viability is maintained throughout the process (8 hours maximum).

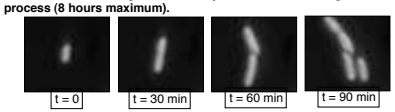


Figure 3. Series of darkfield images of one Klebsiella pneumoniae clone through two doublings.

On-device microorganism viability is maintained throughout the EKC and immunostaining process.

Results 4: Growth rate quantitation

Image processing algorithms identify bacterial clones via threshold discrimination, and track growth by tabulating integrated intensity through a timed sequence of dark field images. Growth constants and doubling times of individual clones are derived from curve fit analysis, and can be expressed as either individualized or aggregate subpopulation values.

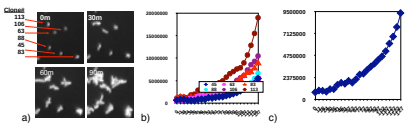


Figure 4. Series of darkfield images of Acinetobacter baumannii showing growth over 90 minute period, with software derived clonal analysis (a). Panel b shows integrated intensity curves of complete dataset of 6 clones identified. Panel c shows growth curve derived from average integrated intensity values from each time point.

Table 1. Exponential growth curve constants and doubling times of 6 tracked clones of A baumannii. Average value derived from means of individual time points.

Clone ID	k (min^{-1})	R^2	DT (min)
45	0.0169	0.917	40.9
63	0.0174	0.973	39.9
83	0.0175	0.962	39.5
88	0.0175	0.967	39.5
106	0.024	0.983	28.8
113	0.0215	0.972	32.3
Average	0.018	0.986	38.6

Growth rate measurements on individual clones in real time

Results 5: Growth rate quantitation comparison

Standard methods of bacterial growth rate quantitation measure OD_{600} changes in a growing suspension culture. The experimental system was used to determine the aggregate growth rates of a panel of ten clinically relevant bacterial species, with the results compared to suspension culture measurements. Average difference of 18% sd 5% demonstrates a high degree of concordance in the methods.

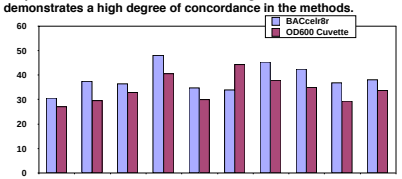


Figure 5. Doubling times in minutes of ten bacterial strains, measured with the BACcelr8r™ and by OD_{600} change in broth culture suspension. Each value is the average of minimum three runs, with each run consisting of four replicates.

Flowcell growth rate measurements parallel standard methods

Results 6: Clinical specimen model

***Sheep Bronchoalveolar Lavage (BAL) with bacterial* spikes provide clinical sample model. Viable microorganisms are recovered from BAL specimen using a simple centrifugation protocol. The sample is resuspended in EKC buffer and run through the instrument, and bacteria recovery is verified with fluorescent detection.**

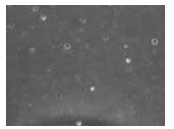


Figure 6a. Phase microscopy image of sheep BAL spiked with 10^8 CFU/mL S. aureus after electrokinetic concentration to capture surface.

Figure 6b. Fluorescence image (same field of view) after antibody identification. S. aureus cells are labeled while BAL debris is not.

Immunochemical bacterial identification shows minimal cross-reactivity with BAL debris components.

Results 7: Antibiotic kill

Individual bacterial clone growth tracking enables evaluation of antibiotic susceptibility and resistance characteristics on a clonal basis within hours.

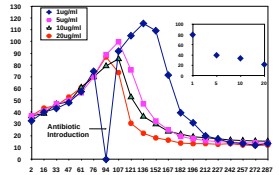


Figure 7. Growth and Kill curves of a set of experiments in which Klebsiella pneumoniae was concentrated, grown, and dosed with varying concentrations of imipenem, a lytic antibiotic. Tracking the decrease in integrated intensity after introduction of antibiotic reveals dose-dependent kill rates. Inset graph shows 50% kill time as function of antibiotic concentration.

Antibiotic susceptibility testing on individual bacterial clones with near real time measurement

Conclusions

A device that allows near real time identification and growth analysis of individual bacterial clones has been developed. Benefits of the technology include:

·Much faster bacterial identification than currently practiced.

·Much faster antibiotic susceptibility testing, providing more timely and accurate selection of antibiotic therapy.

·Gives information on thousands of individual unselected clones, rather than small numbers of selected clones.

·Allows analysis of different sub-populations of bacteria within a sample.

·Will be useful for large-scale drug testing and growth kinetic characterization of bacterial clones.

For further information

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