

16 Microcolony Growth Procedures

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Abstract: Microcolony growth procedures have gained recent interest for the following reasons: rapid simultaneous screening for growth of large numbers of bacteria from environmental samples, time-lapse imaging of single-cell growth, cell aging or biofilm studies. Not many of such procedures have been described in sufficient technical detail to be easily reproduced. Here we provide a simple step-by-step procedure, which allows time-lapse imaging of bacterial cell division and cell tracking up to the stage of a monolayered colony of a few 100 cells.

1 Introduction

For a variety of reasons it can be interesting to study microbial growth at the micro- rather than at the macroscale as is typically done in shaken flask suspended cultures, bioreactors, or the like. Numerous studies on bacterial biofilms or colonies have demonstrated conclusively that cells at different spatial locations within the closely packed cellular structure of a biofilm or colony face different growth conditions and can display different activity and phenotypes (Tolker-Nielsen and Molin, 2000). More recently, the idea of phenotypic identity of individual bacteria among clonal populations has been challenged by the discovery and deeper understanding of bistable switches underlying, e.g., competence development or sporulation (Losick and Desplan, 2008), horizontal gene transfer (Babic et al., 2008; Minoia et al., 2008), by cell aging (Stewart et al., 2005), differential killing and resistance development (Balaban et al., 2004; Dhar and McKinney, 2007), or by cooperative behavior (Ackermann et al., 2008) and bet-hedging (Veening et al., 2008). Phenotypic differentiation was also shown to occur among bacteria degrading hydrocarbons in bioreactors (Wiacek et al., 2006), suggesting that many more such phenomena exist. To study individual cellular behavior in populations one needs different tools that allow single-cell distinction, analysis of cellular genealogies, and easy analysis of individual gene expression. Major advances in the use of autofluorescent proteins, fluorescent staining techniques or *in vivo* specific protein labeling techniques have enabled single-cell study of gene expression and subcellular protein localization patterns (Babic et al., 2008; Glaser et al., 1997). Microscale growth experiments on the other hand are very helpful to set up the conditions under which to study individual cell behavior. Combinations of single-cell tracking in microscale growth experiments may well be used to monitor the reaction time of cells to signals and stimuli, such as light, poisons, nutrients, chemical agents, signaling compounds and so on.

Microscale experiments might also be interesting from the perspective of studying growth on traces of carbon compounds that otherwise are too toxic to be applied in large quantities. Microscale growth has already been shown to offer excellent possibilities for large library screening or for screening growth characteristics of large numbers of environmental isolates simultaneously in micro-Petri dishes (Ingham et al., 2007). The procedure we describe below is an example of how colony formation from a single cell up to a few 100 cells can be monitored in real time by microscopic observation, which may be used for further advanced biodegradation studies as well.

2 General Procedure Strategy and Outline

Microcolony growth procedures are designed as such that direct microscopic observations are possible in real-time conditions without disturbance of the specimen. Several examples of

microcolony growth assays exist and we will briefly mention a few of them, while concentrating our technical description on only one. Bacterial biofilm growth assays could actually be considered the first such strategy for real-time microcolony observation, in which the multilayered structure is analyzed during a period of days up to 2 weeks, as a function of cell type, gene mutations or surface material. In combination with time-lapse imaging it is possible to follow cellular division from single cells to a single-layered microcolony, from which individual cell division rates and cell pole age for every individual in the microcolony can be calculated.

A microcolony growth procedure has to start with the choice of material to grow the structure on. As mentioned, continuous flow biofilm growth experiments typically start with a closed chamber with glass cover slips on both sides (Christensen et al., 1999; Tolker-Nielsen and Molin, 2000). Bacterial cells can be inoculated at low density, some of which will attach to the surface, and because of the constant carbon feed from one direction of the flow cell, will develop to a mature biofilm on the surface in a matter of days (Fig. 1). The formation and structure of the biofilm can be examined microscopically from underneath one of the glass cover slips, ideally by confocal laser scanning microscopy, in order to produce multilayered focused images. Biofilm studies profit greatly from the use of fluorescently tagged cells, which permit easier visualization of individual cells in a complex biofilm structure and allow the *in vivo* study of activity of specific cellular promoters in and during biofilm growth. Procedures for continuous flow biofilm formation have been described extensively elsewhere and need not be repeated here (Christensen et al., 1999; Heydorn et al., 2000). To the best of our knowledge, no study has yet tracked the origin and fate of every single cell in a mature biofilm by time-lapse imaging, but theoretically this should be possible.

Other microcolony growth procedures have used porous materials to inoculate cells onto and follow the microcolony development in time. Such porous materials can be bacterial filters or porous aluminum oxides that can be molded to include nanocavities (Ingham et al., 2007). The porous surfaces can then be placed on a medium surface, from which nutrients will diffuse through the support to the cells. Porous nanocavity plates have the advantage that growth of thousands of microcolonies can be followed simultaneously, because they are physically separated from each other. This permits growth analysis, for example, of separated individual bacterial cells in communities, of phenotypic variation upon stress (Ingham et al., 2008), or of large libraries of mutants.

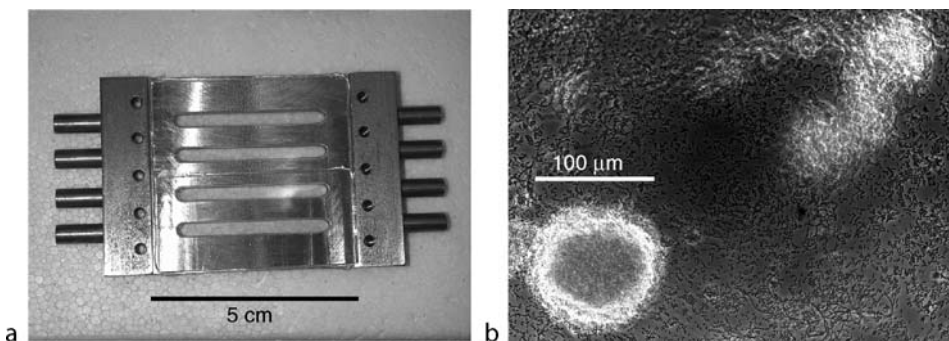


Figure 1

Example of a biofilm flow microcolony growth setup. (a) A four-channel flow chamber, covered on both sides with cover slips. (b) A heterogeneous biofilm of *Pseudomonas knackmussii* B13 on 10 mM 3-chlorobenzoate developed after 5 days flow, imaged at 400× phase contrast.

Microcolony assays have also been carried out in spatially confined microfluidics structures, in which cells are provided with nutrients by diffusion from an agar surface below, but are physically confined to a microstructure on top (Balaban et al., 2004). The microstructure forces the cells to grow within the spatial boundaries defined by the structure, for example, a capillary. In this case cells will divide and arrange themselves in longitudinal direction, which can be used to examine cell arrest, mutant arisal, and phenotypic variation, such as upon antibiotic addition (Balaban et al., 2004).

Finally, microcolonies can be grown on the surface of a semisolidified gel matrix that provides nutrients to the cells, but is so thin that light passes through and cell division can be followed directly by microscopy. In this procedure, cell division is physically constrained by the presence of the gel surface on one and a glass cover slip on the other side. Even so, in our experience two- to three-layered microcolonies will develop eventually, that are not hindered in the *z*-direction by the cover slip. Examples of this procedure include recent studies that have used methods of single-cell tracking (Babic et al., 2008; Stewart et al., 2005; Veening et al., 2008). The crux in the procedure is to form a flat gel surface onto which to inoculate the cells; then cover the surface but still allowing oxygen (for aerobic bacteria) to penetrate, and to prevent drying out of the surface and the cells. Glass slides with small concave inclusions and silicon surrounding ridges have hereto been deployed (Babic et al., 2008; Stewart et al., 2005).

The procedure we describe in more detail below was inspired on earlier gel matrix assays, but with some modifications. These modifications, we find, easily allow further online manipulation of growth conditions or cell staining during the course of an experiment without interfering with the original structure of the microcolonies and the positions of the individual cells therein. Our procedure also allows long-term (up to 1 week) online-monitoring of the cells in the microcolony, without desiccation or oxygen depletion throughout the length of the experiment. The protocol describes the creation of a flat and thin nutrient surface on which cells can be inoculated and are constrained by a glass cover slip, but with the difference that the other side of the gel matrix is exposed to a headspace (► Fig. 2). The system is then placed within an air tight microscope observation chamber for incubation and time-lapse imaging. We termed this protocol the *pancake* method because it involves the turning of a gel patch, similar to the flipping of a pancake (see ► Fig. 2).

Since the gel patch is inoculated with a diluted cell suspension, flipping of the gel patch results in the entrapment of isolated single cells at the glass-gel interface (► Fig. 2a). Nutrients reach the cells by diffusion from the gel, whereas the gel layer is so thin (1 mm) that oxygen can diffuse freely to the cell layer that forms in between the gel and the glass (► Fig. 2b). Further substrate can be added on top of the gel without interfering with the original positions of the cells or developing microcolonies on the other side. Substrate addition can be done either in form of a liquid solution, or as a volatile released into the headspace within the closed chamber (in the case of, e.g., naphthalene or toluene). Additional advantage is that the cells can be specifically stained after defined incubation periods, by applying the dye again on top of the gel surface, after it will diffuse through the gel and stain the cells. Examples of useful stains include the Live/Dead reagent (Invitrogen, Molecular Probes).

Not unsurprisingly, cells grow into microcolonies even without extra added carbon source to the gel, because of carbon traces in the gel that cannot be completely removed. Final average colony sizes even without any carbon added reach up to a few 100 cells, but this size is inversely proportional to the density of the inoculated cell suspension. The concentration of cells in the inoculum can thus easily be adjusted to obtain monolayered microcolonies (► Fig. 3), which is an important prerequisite when single cells are to be investigated with microscopes that cannot

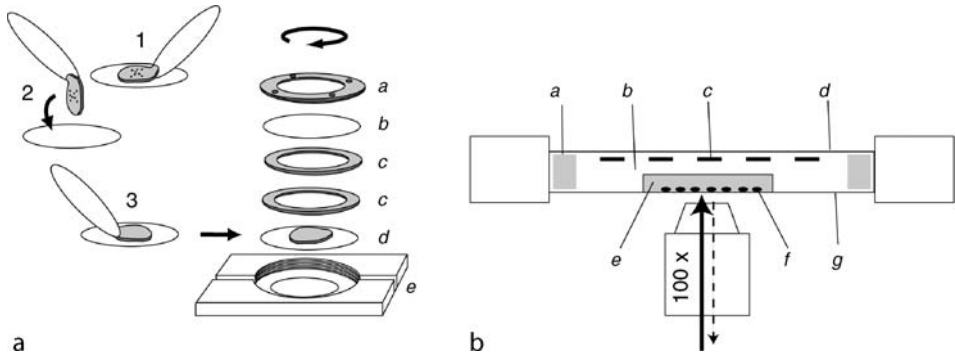


Figure 2

Schematic outline of the pancake method and perfusion incubation chamber assembly.

(a) POC assembly parts and gel patch flipping. a: Locking ring, b: upper coverslip, c: silicon ring, d: lower coverslip, cells, and gel patch, e: perfusion chamber. (b) Sideview of POC chamber in inverted microscope configuration. a: Silicon ring, b: headspace, c: condensation, d: upper coverslip, e: gel patch, f: cells, g: lower coverslip, h: objective.

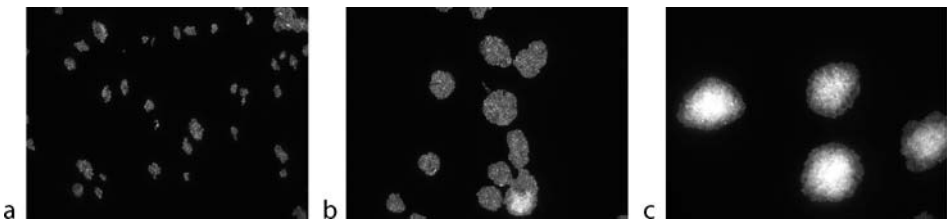


Figure 3

Microcolony sizes of *Pseudomonas putida* UWC1-ICEIc equipped with a constitutively expressed mCherry protein after 144 h incubation at 20°C on agarose surfaces supplemented with 0.1 mM 3-chlorobenzoate in the pancake method. Initial inoculation densities: (a) $\approx 10^4$ cells μl^{-1} , (b) $\approx 1,000$ cells μl^{-1} , (c) ≈ 100 cells μl^{-1} . Images taken in mCherry epifluorescence and upright mode with a Zeiss AxioScope at 400 \times magnification.

resolve along the z-axis (like confocal laser scanning microscopy, CSLM), or in experiments with prolonged incubation periods. In our hands, we find that at above 0.1 mM extra carbon added to the gel, colony sizes will start to increase as a result of the specific extra carbon source.

We generally assemble the whole setup in a commercially available *perfusion chamber system* from H. Sauer (Germany, see below, [▶ Fig. 2](#)), which comes with a rigid metal cast POC chamber, round coverslips and silicon or Teflon spacer rings. The POC chamber allows both closed and open configurations, and several add-on options for nutrient-flow circuits exist. But in theory any airtight system (to prevent desiccation of the gel patch) consisting of two glass cover slip layers and a spacer-ring(s) should work as long as it fits into the microscope table.

We used the pancake method to full satisfaction in combination with three different microscopes with different setups, a Zeiss AxioScope upright epifluorescence microscope, a Leica inverted epifluorescence microscope, and an inverted CLSM from Leica. CLSM has the obvious advantage that multilayered cell structures can be focused more properly, but has

the disadvantage that quantification of autofluorescent protein signals is more difficult (in case this is important). In the upright microscope-POC chamber configuration, the distance of the chamber to the condenser is too large to enable proper phase-contrast imaging, but the epifluorescence imaging mode is unrestricted.

3 Advantages and Disadvantages

We have experienced the following advantages of our modified procedure to prepare gel-surface growth in a closed microscope chamber. First, because of the system containment and upside-down configuration, the gel surface is less prone to shrinking in the z-direction because of desiccation. We found that in the “cells-on-top” configuration, an agarose surface (even in a completely closed chamber with moisture) shrinks with about $1\text{--}2\ \mu\text{m day}^{-1}$, which makes automated imaging more difficult except if z-stacks are recorded. In the upside-down configuration, any shrinking of the agar surface does not influence the automated focusing of cells directly close to the glass cover slip (► *Fig. 2b*). Secondly, the use of the microscope chamber with round cover slips and upside-down configuration permits the proper functioning of even short working distance lenses, which are basically limited to the thickness of the cover slip (0.17 mm) at high magnification. Thirdly, in upside-down configuration on an inverse microscope, the fluorescence light path does not travel through the agarose nor is it obstructed by condensation on the upper glass cover. Turning the POC chamber around for observation on an upright microscope has the small risk that the gel patch will fall down or loosen because of gravity. Condensation cannot form between the agarose patch and bottom coverslip. Sequential carbon substrates or staining dyes can be applied on the gel surface without affecting or disrupting the positions of single cells or the microcolony structure. In combination with CLSM multilayered colonies can be explored. The method is suitable for single-cell tracking, time-lapse imaging, and single-cell fluorescence imaging over periods of 1 week. Finally, the microscope chambers can be positioned exactly on the microscope stage, but also removed and incubated separately, if multiple such systems need to be analyzed and imaged.

The pancake method does have some drawbacks. Turning around the gel patch requires a bit of practice. As an alternative the second cover slip can be placed directly on the inoculated cell surface, and then turned around, but we find that in that case removing the support cover slip for the gel (which is now on the top side) is not unproblematic and can either break or will again lift the whole patch from the lower glass cover. Condensation forms on the inside of the upper coverslip obstructing somewhat bright-field and phase-contrast views, both of which rely on light passing through the whole chamber from the opposing light source into the lens. Another disadvantage of the gel patch protocol might be accumulation of (toxic) metabolites and change of pH over time in the gel surface provided to the cells.

4 Procedure

4.1 Protocol: Preparation of the Gel Patch

Prepare the POC Chamber and the cover slips by autoclaving and subsequent drying in a sterile flow hood. Separate the cover slips during autoclaving by inserting a tooth pick between them. Install a round cover slip (42 mm diameter, 0.17 mm thickness) at the bottom of the

POC chamber and place a 1 mm Silicon ring on top (► [Fig. 2](#)). For optimal sterile conditions carry out the whole procedure in a laminar air flow bench.

1. Place a drop of 150 μl molten liquid 1% agarose medium maintained at 50°C on the bottom glass cover slip.
2. Immediately place another round cover slip on top of the agarose medium droplet. Press softly until the droplet is flat and the top cover slip touches the Silicon separation ring. Let the agarose solidify.
3. Remove the upper glass slide and the Silicon ring to expose the agarose surface.
4. Prepare the bacterial suspension to inoculate on the surface. The cell density in this suspension may depend on the research question, but we generally start with a suspension that has $\approx 5 \cdot 10^6$ cells ml^{-1} . This corresponds approximately to a 100-fold dilution of an overnight culture on 4 mM fructose. Collect the cells by centrifugation at low speed and wash once in carbon free minimal medium to remove traces of organic carbon or possible toxic secondary metabolites. Centrifuge again to collect cells and resuspend in the medium to the final desired cell density.
5. Pipette a volume of 10 μl of cell suspension on the planar agarose surface, distribute the droplet evenly by tilting in a circular motion and allow it to dry at room temperature in the sterile flow hood. This typically takes 5–10 min. Stop when a water film is no longer visible. Avoid drying too long or else the agarose will dry out.
6. Now take another coverslip to lift the agarose patch and place it upside down onto a new round cover slip for the POC chamber. This requires some practice but is most easily performed by slightly inserting the edge of a cover slip at an angle between the agarose and the support glass surface until the agarose makes enough contact with the new coverslip to be lifted (► [Fig. 2a](#): steps 1–3). The cells will now be facing down.

Note: As an alternative, you could add a new cover to the side of the gel patch with the cells, turn the system around and remove the back support cover slip. We find, however, that this is more difficult because the back cover often sticks better to the agarose.

7. Insert the new support coverslip with the gel patch into the POC chamber. Now place two Silicon rings (each of 1 mm thickness), finally a fresh round glass cover and close the system with the metal ring.

Note: Instead of placing the gel patch in a closed configuration, the POC chamber can also be operated in open mode, which makes subsequent manipulation of substrates or dyes more easy. The open POC-mode requires, however, an inverted microscope setup.

8. The chamber is now ready to be mounted on a microscope. Growth temperature can be adjusted to ambient temperature of the room in which the microscope is operated, or set to a specific temperature if an incubation chamber on the microscope table is used.

Note: As an alternative, the POC chamber can be removed from the microscope table for external incubation in a small temperature incubator. In this case, one cannot make time-lapse imaging and finding back the exact position may be more difficult.

9. It may be fairly difficult at first to spot and focus single cells at the agarose-cover slip interface. It is helpful to find the edge of the gel patch and use this area to focus, because often a higher concentration of bacteria can be found here. From there on move towards the center of the patch and focus on an isolated single cell. To make sure to focus on a

growing individual cell, wait until the first cell division before setting up the automated time-lapse function.

10. For time-lapse microscopy take images at regular time intervals. The shorter the time interval the more accurate every cell can be followed. Cells will start to divide synchronously but after a few divisions will lose this synchronicity. Typically we start with images taken every 10–15 min. Manual readjustments may be needed to remain in focus over longer periods. Depending on programming abilities and automated x - y stages, multiple positions can be monitored over time.
11. We use Metamorph or Metaview (Molecular Devices) to produce image stacks and track cells, but any imaging program can be of help here. ImageJ is a public domain software for image analysis (rsb.info.nih.gov/ij/).

4.2 Optional Protocol: Cell Staining and New Carbon Substrates

We find that microcolonies grown to stationary phase on gel patches can be reactivated by the addition of new substrate. For this purpose, open of the POC chamber and pipette a 10 μ l droplet of a 100 mM carbon substrate solution on the top of the gel patch. Within 15 min the substrate has reached the cells by diffusion.

In a similar manner, dyes can be added by opening the POC chamber and applying a 10 μ l drop of the dye solution (e.g., Live-Dead[®] stain from Invitrogen) on the top of gel patch. Dyes will diffuse through the cell and stain the cells after \approx 15 min. Staining is complete after 1–2 h. Do not add more than 10 μ l at once, or else microcolonies can disassemble at the glass-gel interface.

5 Materials

5.1 Chemicals and Solutions

Growth medium of choice for your favorite bacterium. For *Pseudomonas*, *Burkholderia* and *Cupriavidus* we use *Pseudomonas* defined medium (Gerhardt et al., 1981). The basic medium contains per liter: 1 g NH_4Cl , 3.49 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.77 g KH_2PO_4 , has a pH of 6.8 and is sterilized by autoclaving. Carbon substrates are added according to the experimental setup. Afterwards per liter of medium 20 ml of Hutner's vitamin free mineral base (filter sterilized), and 2 ml of a 500-fold diluted filter-sterile vitamin solution are added. Hutner's vitamin free mineral base contains per liter: 10 g NTA, dissolve and neutralize with ca. 6 g KOH, 14.45 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.33 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 9.74 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 99 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 50 ml of Metals 44 solution. Hutners is sterilized by filtration and stored 4°C. Metals 44 solution contains per 100 ml: 387 mg $\text{Na}_4\text{EDTA} \cdot 4\text{H}_2\text{O}$, 1.095 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 914 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 154 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 39.2 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 24.8 mg $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 17.7 mg $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, a few drops of 6 N H_2SO_4 , is sterilized by filtration and stored at 4°C. Antibiotics can be added upon desire and according to the characteristics of the bacterial strain used.

Agarose medium solution is prepared by dissolving 1.0% (w/v) ultrapure agarose into the selected growth medium. Autoclave the growth medium with the agarose, cool down to 50°C before pouring the gel patch.

5.2 POC Chamber

We use a POC setup (Helmut Saur Laborbedarf, D-72734 Reutlingen, Germany). Silicon rings come with the system. Teflon[®] rings can be custom manufactured to resist organic volatile contaminants if used as growth substrate. The system can be operated with round coverslips, 42 or 25 mm ϕ (Helmut Saur).

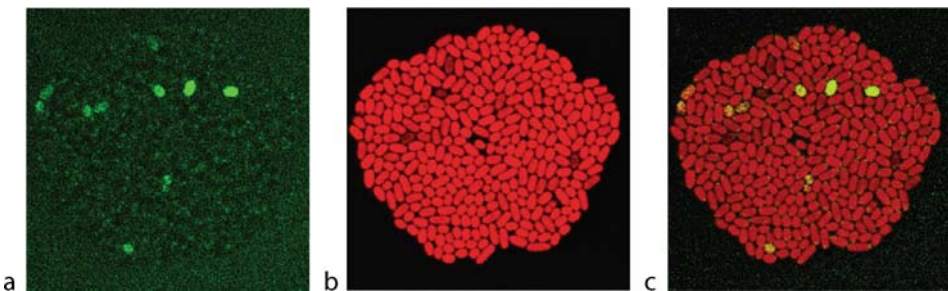
5.3 Microscope

Epifluorescence microscope (upright or inverse) with appropriate filters in case fluorescent markers are used. Confocal laser scanning microscope for analysis of multiple cell layers. Magnifications: $63\times$ or 100×10 . Preferentially use objectives with long working distances.

6 Data Examples

6.1 Example 1. Quantification of Microcolony GFP Expression

In this example we used a *Pseudomonas putida* strain constitutively expressing mCherry from the *tac* promoter, and *egfp* from the integrase promoter *intB13p* (Minoia et al., 2008). Microcolonies were grown on gel patches with 0.1 mM 3-chlorobenzoate to study induction of the P_{int} promoter in stationary phase. These conditions are known to result in bifurcation of the population and leading to around 2–5% of cells expressing P_{int} (Sentchilo et al., 2003). Microcolonies were grown from single cells in the pancake protocol for up to 1 week. After 24 h cells in the colony stopped dividing and the cells entered stationary phase. The overlay image (► Fig. 4) shows *egfp* expression in a number of individual cells in a microcolony of cells that all express mCherry at 48 h after start of the experiment.



► Figure 4

Example of a microcolony of *Pseudomonas putida* UWC1-ICElc constitutively expressing mCherry and inducing *egfp* from *intB13p*, formed on agarose surfaces with 0.1 mM 3-chlorobenzoate after 48 h. (a) Confocal laser scanning image in excitation and emission wavelengths for *egfp* at $1,000\times$ magnification. (b) Idem for mCherry. (c) Overlay of A and B. Note that only a small proportion of cells express *egfp* from *intB13p*.

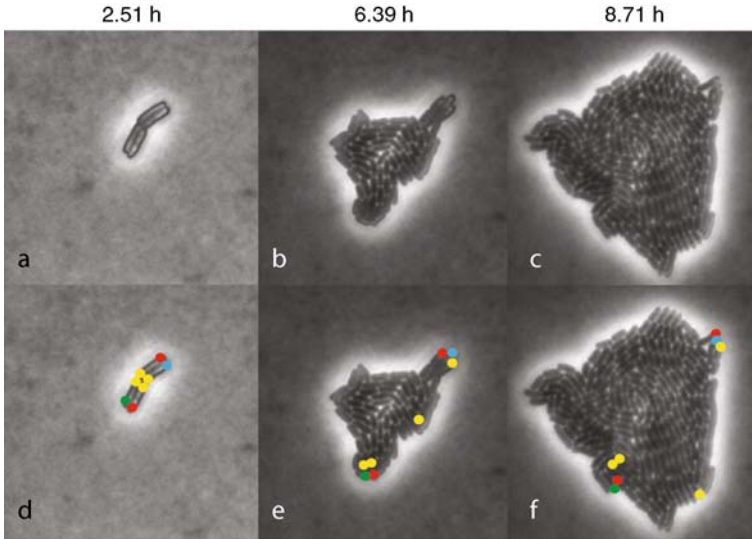


Figure 5

Cell pole tracking in individuals within a developing microcolony of *Cupriavidus necator* on diluted agarose medium in the pancake method. (a) Four-cell stage after 2.51 h. (b) and (c) Multiple cell stages after 6.39 and 8.71 h, respectively. (d–f) Corresponding marker overlays to track poles. Ancestral cell poles: green and blue. Second generation new cell poles: red. Third generation cell poles, yellow.

6.2 Example 2. Cell Pole Tracking Using Time-Lapse Fluorescence Microscopy

Cell pole tracking is a technique that is used to study bacterial cell aging effects. In this example we used *Cupriavidus necator* strain JMP134, a strain which degrades 2,4-dichlorophenoxyacetic acid (Laemmli et al., 2000), but which was grown here on diluted nutrient broth agarose gel patches. Microcolony development was followed here by phase-contrast microscopy with images taken every 10 min. The three images show manual tracking of young cell poles appearing in the first, second, and third generation of cell division over the time course of seven generations (8.71 h, ▶ Fig. 5).

7 Outlook and Research Needs

There is a clear new interest in single-cell microbiology, phenotypic variation and its underlying causes. As outlined above this requires sets of methods to visualize and record the fates of individual bacteria during growth. Although a number of simple methods to grow and follow individual bacterial growth exist currently, many more will be deployed by combining microstructuring and – fluidics techniques with bacteria growth. Advances in computer systems and microscopy will doubtless permit next generation data recordings, enabling further dissection and understanding of microbial life and development at the real microscale level.

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