

particular metabolite, enzyme, protein, nucleic acid, phenotype, mutation, metabolic pathway, gene, adaptation, capability, and/or therapeutic benefit is challenging, requiring complex and expensive methods. For example, microbes live in extremely high-risk environments. To survive, microbes have developed amazing sets of biochemical tools, including novel enzymes, unique metabolites, innovative genetic pathways, and strategies for manipulating their environment and their microbial neighbors – powerful solutions that could lead to new insights and products ranging from life-saving antibiotics to fertilizers that improve food production and security.

SUMMARY

The present disclosure provides microbiology systems, apparatus, kits, and methods for streamlining the cultivation workflow, supporting high throughput screening, and/or developing new insights and products in accordance with some examples. For example, an apparatus may comprise a microfabricated device for receiving a sample comprising one or more cells. The microfabricated device defines a high density array of microwells (sometimes referred to as “wells” for simplicity) for cultivating one or more cells.

Thus, in one aspect, the present disclosure refers to a method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, the method comprising: loading into at least one microwell of the array of microwells: (a) at least one cell from the sample; (b) an indicator substance which comprises a pH sensitive dye; and (c) an amount of a nutrient; applying a membrane to the microfabricated device to retain the at least one cell in the at least one microwell; incubating the microfabricated device at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell; evaluating an optical property of the at least one microwell after incubation; and determining a presence or absence of at least one biological entity of interest that has produced an acid influencing the optical property in the at least one microwell during incubation based on the optical property.

In another aspect, the present disclosure refers to a method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, the method comprising: loading into at least one microwell of

the array of microwells: (a) at least one cell from the sample; (b) an indicator substance which comprises a pH sensitive dye; and (c) an amount of a nutrient; applying a membrane to the microfabricated device to retain the at least one cell in the at least one microwell; incubating the microfabricated device at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell; and determining a presence or absence of at least one biological entity of interest that has produced an acid in the at least one microwell during incubation based on a change of the indicator substance after incubation.

In yet another aspect, the present disclosure refers to a method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, wherein each of the microwells has a bottom, the method comprising: depositing an indicator substance which comprises a pH sensitive dye into each of a plurality of microwells of the array of microwells; loading the sample onto the microfabricated device such that at least one microwell of the plurality of microwells receives at least one cell from the sample and an amount of a nutrient; applying a membrane to the microfabricated device to retain the received at least one cell and the nutrient in each of the plurality of microwells; incubating the microfabricated device at predetermined conditions for a duration of time; and determining a presence or absence of at least one biological entity of interest that has produced an acid in the at least one microwell of the plurality of microwells during incubation based on a measurement of an optical property of the at least one microwell after incubation, wherein the optical property is influenced by the acid.

In some examples, a device with a high density array of microwells for cultivating cells may be provided. In further examples, a set of unique tags may be provided for disposal into each microwell or some of the microwells. Alternatively, the device may be provided with the unique tags already seeded into each microwell or some of the microwells. The device and/or the unique tags may be provided alone or part of a kit. For example, a kit may include a microfabricated device for receiving a sample comprising one or more cells, the microfabricated device defining a high density array of microwells for cultivating the cell(s). A kit also may include a set of unique tags for disposing in the microwells so as to identify the specific microwell in which a species of cell is cultivated. One or more unique tags may be disposed in each microwell of the high density array of microwells. A kit further may include instructions for seeding microwells with unique tags and/or using the unique tags to trace cultivated cells back to the specific microwell in which they were cultivated.

In some examples, a unique tag may include a nucleic acid molecule with a target-specific nucleotide sequence for annealing to a target nucleic acid fragment of a cell or a particular species of cell (e.g., an organism) that may be present in a microwell and a location-specific nucleotide sequence for identifying the microwell itself. More than one unique tag may be disposed in a particular microwell such that more than one location-specific nucleotide sequence is present in the particular microwell. That is, the location-specific nucleotide sequences may be found in more than one microwell (e.g., predetermined to identify a dimension of microwells in the high density array of microwells), and the unique tags may be multi-dimensional. For example, a set of unique tags may be two-dimensional, including tags with a first location-specific nucleotide sequence predetermined to identify a first dimension of microwells (e.g., a row) in a high density array of microwells and tags with a second location-specific nucleotide sequence predetermined to identify a second dimension of microwells (e.g., a column) in the high density array of microwells. Together, the first location-specific nucleotide sequence and the second location-specific nucleotide sequence identify a specific microwell of the high density array of microwells. Thus, the combination of unique tags in a particular microwell operates to identify that microwell.

In some examples, one or more nutrient may be provided for growing and/or screening cells within each or some of the microwells of a device including a high density array of microwells for cultivating cells. One or more nutrients may be provided for disposal in all or some of the microwells (e.g., directly in a microwell, through a membrane, and/or on a permeable or semi-permeable membrane. Alternatively, the device may be provided with one or more nutrients already disposed in each microwell or some of the microwells. A nutrient may be provided alone, as part of a medium, and/or as part of a kit. A kit may include instructions for providing one or more nutrients to cells being cultivated within specific microwells in the device. For example, a kit further may include at least one nutrient for cultivating the at least one species of the at least one cell. The at least one nutrient for cultivating the at least one species of the at least one cell may include or be a component of an extract from a natural environment of the at least one species of the at least one cell, a medium derived from the natural environment of the at least one species of the at least one cell, a medium formulated to resemble the natural environment of the at least one species of the at least one cell, a selective medium to cultivate only the at least one species of the at least one cell, and/or a differential medium to distinguish the at least one

species of the at least one cell. The natural environment of the at least one species of the at least one cell may be a biological tissue, a biological product, a microbial suspension, air, soil, sediment, living organic matter, forage, petroleum, sewage, and/or water.

5 In some examples, a membrane may be provided for keeping cells within each or some of the microwells (e.g., preventing cross-contamination) of a device including a high density array of microwells for cultivating cells. A membrane may be applied to seal some or all of the microwells. Alternatively, the device may be provided with the membrane already sealed over some or all of the microwells. A membrane may be, for example, impermeable, permeable only by gas, or allow for diffusion of one or more nutrients into one or more microwells. The device
10 and/or the membrane may be provided alone or as part of a kit. For example, a kit may include a microfabricated device for receiving a sample comprising at least one cell. A kit also may include a membrane to retain the at least one cell in the at least one microwell of the high density array of microwells after the sample is loaded on the microfabricated device. A kit further may include instructions for loading microwells with a sample and/or using the membrane keep
15 cultivated cells within the specific microwells in which they were cultivated.

In some examples, steps are provided for cultivating cells using a device with a high density array of microwells for cultivating cells. For example, a method may include obtaining a microfabricated device defining a high density array of microwells for cultivating at least one cell from a sample, which may include disposing at least one unique tag in at least one microwell
20 of the high density array of microwells, loading the device with a sample including one or more cells, and/or applying a membrane to all or a portion of the device to retain the cells in their respective microwells. In some examples, a sample is prepared before loading. For example, a sample may be diluted such that no more than one cell is disposed in each microwell of a preponderance of the high density array of microwells. A method also may include incubating a
25 device to grow a plurality of cells from the at least one cell in the at least one microwell of the high density array of microwells.

In some examples, steps are disclosed for replicating and/or splitting cells cultivated in a high density array of microwells such that a portion of the cells may be modified and/or destroyed for analysis (e.g., amplified and sequenced) whereas the remaining cells may be

reserved for future sampling and/or use based on the information gleaned from the analysis. For example, a method may include splitting a plurality of cells in the at least one microwell of the high density array of microwells into a first portion of the plurality of cells and a second portion of the plurality of cells. The at least one unique tag may remain with at least the first portion of the plurality of cells. Splitting the plurality of cells in the at least one microwell of the high density array of microwells may include removing the membrane from the at least one microwell of the high density array of microwells such that some of the cells remain in the at least one microwell of the high density array of microwells and some of the cells remain on the membrane. Alternatively, a membrane may be temporarily peeled back to allow sampling or picked through (i.e., punctured) for sampling.

In some examples, steps are disclosed for analyzing all or some of the cells cultivated in a high density array of microwells. For example, a method may include analyzing a first portion of a plurality of cells to determine at least one species of interest. The cells to be analyzed may be in the same microwell in which they were cultivated, attached to a membrane that was covering the microwell in which they were cultivated, and/or transferred to another location (e.g., a petri dish or a second device with a corresponding high density array of microwells). Analysis may include determining a presence or an absence of at least one species of interest, which may be selected based on at least one of a nucleic acid and a gene. For example, a polymerase chain reaction (PCR) may be performed to amplify any of the target nucleic acid fragments present in the cells based on the target-specific nucleotide sequence of the at least one unique tag. Analysis may further comprise the step of sequencing any PCR-amplified target nucleic acid fragments using next generation sequencing (NGS) or any other type of sequencing. Sequencing may be used to identify a species of cell (e.g., an organism) and/or one or more locations in the original high density array of microwells at which the species of cell was cultivated.

In some examples, steps are disclosed for identifying and/or locating one or more microwells in a high density array of microwells in which a particular species of cell was cultivated. For example, a method may include identifying at least one microwell of the high density array of microwells in which at least one species of interest was cultivated based on at least one unique tag. Based on identifying the at least one microwell of the high density array of microwells in

which the at least one species of interest was cultivated, a method may include locating the at least one species of interest within the second portion of the plurality of cells.

In some examples, steps are disclosed for picking or sampling one or more microwells in a high density array of microwells in which a plurality of cells were cultivated. For example, a method
5 may include picking at least one cell from a particular microwell or a membrane location correlated to the particular microwell. Devices and methods are described for picking from more than one microwell simultaneously.

In some examples, methods are disclosed for screening cells using a device with a high density array of microwells for cultivating cells. For example, a method may include screening cells
10 cultivated in a high density array of microwells to determine a presence or an absence of a species of interest. If present, the method may include picking one or more cells of the species of interest from one or more microwells in which the cells were cultivated. A method may include performing an assay based on a metabolite, a metabolic pathway, an enzyme, a protein, a nucleic acid, a phenotype, a gene, a mutation, an adaptation, and/or a capability of the cells. If
15 unique tags were utilized, a method may include using one or more location-specific nucleotide sequences to identify one or more microwells in which a species of interest was cultivated. For example, a target nucleic acid fragment may be present in the species of interest, the target nucleic acid fragment including genetic code relating to a metabolite, a metabolic pathway, an enzyme, a protein, a nucleic acid, a phenotype, a gene, a mutation, an adaptation, and/or a
20 capability.

In some examples, methods are disclosed for determining a relative abundance and/or an absolute abundance of a species of interest in a sample using a device with a high density array of microwells for cultivating cells. For example, a method may include preparing and loading a sample into microwells with a set of unique tags disposed therein, the sample prepared such that
25 no more than one cell of the at least one cell is disposed in each microwell of a preponderance of the high density array of microwells. Following cultivation, a method may include using the tags to determine a first number of microwells with at least one cell, a second number of microwells with the species of interest, and based thereupon, a relative abundance of the at least one species of interest in the sample. A method may further include determining a total number of cells from

the sample and, based on the total number and a relative abundance of at least one species of interest in the sample, an absolute abundance of the at least one species of interest in the sample.

More generally, systems, apparatus, kits, and methods are disclosed for cultivating a biological entity from a sample using a microfabricated device defining a high density array of

5 experimental units. A biological entity may be an organism (e.g., a microorganism such as bacteria or fungi), a cell (e.g., a eukaryotic cell), a cell component, a cell product, and a virus. In some examples, more than one biological entity may be present in a sample, loaded on the device, and/or loaded in an experimental unit. An experimental unit may include one or more biological entities and/or one or more unique tags to identify spatial information relating to the
10 particular experimental unit. A unique tag may include a binding molecule, such as a protein, a nucleic acid, a carbohydrate, a lipid, and/or a drug. For example, a unique tag may include a target-specific component for binding to a biomolecule present in a biological entity of interest. A unique tag also may include a location-specific component for identifying spatial information relating to the at least one experimental unit. The device may be incubated to grow a plurality of
15 biological entities in the high density array of experimental units. One or more cultivated biological entities may be analyzed to identify one or more species and/or spatial information relating to one or more experimental units in which the entities were cultivated based on the at least one unique tag.

In some examples, a method of screening for at least one biological entity (e.g., bacteria cells,
20 eukaryotic cells) in a sample is provided. The method employs a microfabricated device as described herein that has a top surface defining an array of microwells. At least one microwell of the array of microwells is loaded with: (a) at least one cell from a sample; (b) an indicator substance; and (c) an amount of a nutrient. The cell, the indicator substance, and the nutrient can be loaded sequentially, in any order, or any of their combinations can be loaded sequentially of
25 simultaneously. A membrane is applied to the microfabricated device to retain the at least one cell in the at least one microwell. The microfabricated device is incubated at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell. An optical property, e.g., color, fluorescence, phosphorescence, or luminescence of the at least one microwell can be evaluated. This evaluation can be done at the completion of the
30 incubation, and/or at multiple points in time, e.g., after loading the microwell

contents but before incubation, and after the incubation has begun but has not finished, etc.

Based on the optical property evaluated or the comparison across multiple times of evaluation of the optical property, a presence or absence of at least one biological entity of interest in the at least one microwell can be determined.

- 5 In some examples, the optical property of the at least one microwell correlates to the amount or form of the indicator substance.

In some examples, the indicator substance comprises an inorganic phosphate salt that is insoluble in water. In some of these examples, the biological entity of interest can comprise a microorganism that solubilizes the inorganic phosphate salt.

- 10 In some examples, the indicator can be a pH sensitive dye. For example, the pH sensitive dye can change color in a visible light range within a selected range of pH. Alternatively, the pH sensitive dye can be photoluminescent (fluorescent or phosphorescent) in a first range of pH and not photoluminescent in a second, different range of pH, or a dye whose photoluminescence changes at different pH.

- 15 In some examples, the at least one biological entity of interest can comprise a microorganism that produces an acid.

In some examples, measuring the optical property comprises taking a plurality of measurements of the optical property at different times during the course of the incubation.

- 20 In some examples, the method further comprises transferring at least some of the plurality of cells after incubation to a target location if a biological entity of interest is determined to be present in the at least one microwell.

In some examples, the at least one microwell includes a plurality of microwells, and loading the at least one cell comprises loading into each of the plurality of microwells, on average, one cell.

- 25 In some examples, each microwell of the array of microwells has a diameter of about 25 μm to about 500 μm . In some examples, the surface density of the array of microwells is at least 750 microwells per cm^2 .

In some examples, evaluating the optical property comprises: (a) measuring the optical property of the at least one microwell after the at least one cell, the indicator substance, and the

nutrient have been loaded and before incubation; (b) measuring the optical property of the at least one microwell after incubation; and (c) comparing the measured optical property before incubation and after incubation.

In some examples, a method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, is provided. The method comprises: loading into at least one microwell of the array of microwells: (a) at least one cell from the sample; (b) an indicator substance; and (c) an amount of a nutrient; applying a membrane to the microfabricated device to retain the at least one cell in the at least one microwell; incubating the microfabricated device at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell; and determining a presence or absence of at least one biological entity of interest in the at least one microwell based on a change of the indicator substance after incubation.

In some examples, a method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, is provided. The method comprises: depositing an indicator substance into each of a plurality of microwells of the array of microwells; loading the sample onto the microfabricated device such that at least one microwell of the plurality of microwells receives at least one cell from the sample and an amount of a nutrient; applying a membrane to the microfabricated device to retain the received at least one cell and the nutrient in each of the plurality of microwells; incubating the microfabricated device at predetermined conditions for a duration of time; and determining a presence or absence of at least one microorganism biological entity of interest in the at least one microwell of the plurality of microwells based on a measurement of an optical property of the at least one microwell.

It should be appreciated that all combinations of the foregoing concepts and additional concepts discussed in greater detail below (provided such concepts are not mutually inconsistent) are contemplated as being part of the inventive subject matter disclosed herein. In particular, all combinations of claimed subject matter appearing at the end of this disclosure are contemplated as being part of the inventive subject matter disclosed herein. It should also be appreciated that terminology explicitly employed herein that also may appear in any disclosure incorporated by reference should be accorded a meaning most consistent with the particular concepts disclosed

herein.

Other systems, processes, and features will become apparent to those skilled in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, processes, and features be included within this description, be within the
5 scope of the present disclosure, and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The skilled artisan will understand that the drawings primarily are for illustrative purposes and are not intended to limit the scope of the inventive subject matter described herein. The drawings are not necessarily to scale; in some instances, various aspects of the inventive subject
10 matter disclosed herein may be shown exaggerated or enlarged in the drawings to facilitate an understanding of different features. In the drawings, like reference characters generally refer to like features (*e.g.*, functionally similar and/or structurally similar elements).

FIG. 1 is a perspective view illustrating a microfabricated device or chip in accordance with some embodiments.

15 FIGS. 2A-2C are top, side, and end views, respectively, illustrating dimensions of microfabricated device or chip in accordance with some embodiments.

FIGS. 3A and 3B are exploded and top views, respectively, illustrating a microfabricated device or chip in accordance with some embodiments.

FIGS. 4A and 4B are diagrams illustrating a membrane in accordance with some embodiments.

20 FIG. 4C is an image of a membrane surface with impressions formed from contact with an array of wells in accordance with some embodiments.

FIG. 5A is a flowchart illustrating a method for isolating cells from a sample in accordance with some embodiments. FIG. 5B is a diagram illustrating a method for isolating cells from a soil sample in accordance with some embodiments.

25 FIG. 6 is a flowchart illustrating a method for isolating and cultivating cells from a sample in accordance with some embodiments.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, the method comprising:

loading into at least one microwell of the array of microwells: (a) at least one cell from the sample; (b) an indicator substance which comprises a pH sensitive dye; and (c) an amount of a nutrient;

applying a membrane to the microfabricated device to retain the at least one cell in the at least one microwell;

incubating the microfabricated device at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell;

evaluating an optical property of the at least one microwell after incubation; and

determining a presence or absence of at least one biological entity of interest that has produced an acid influencing the optical property in the at least one microwell during incubation based on the optical property.

2. The method of claim 1, wherein the optical property of the at least one microwell correlates to the amount or form of the indicator substance.

3. The method of claim 1, wherein the pH sensitive dye can change color in a visible light range within a selected range of pH.

4. The method of claim 1, wherein the pH sensitive dye is fluorescent in a first range of pH and not fluorescent in a second, different range of pH.

5. The method of claim 1, wherein the pH sensitive dye is a fluorescent dye whose fluorescence changes at different pH.

6. The method of claim 1, wherein the at least one biological entity of interest comprises a eukaryotic cell.

7. The method of claim 1, wherein the at least one biological entity of interest comprises bacteria.

8. The method of claim 1, wherein measuring the optical property comprises taking a plurality of measurements of the optical property at different times during the course of the incubation.

9. The method of claim 1, further comprising:
if the biological entity of interest is determined to be present in the at least one microwell, transferring at least some of the plurality of cells after incubation to a target location.

10. The method of claim 1, wherein the at least one microwell includes a plurality of microwells, and wherein loading the at least one cell comprises loading into each of the plurality of microwells, on average, one cell.

11. The method of claim 1, wherein each microwell of the array of microwells has a diameter of 25 μm to 500 μm .

12. The method of claim 1, wherein the surface density of the array of microwells is at least 750 microwells per cm^2 .

13. The method of claim 1, wherein evaluating the optical property comprises:

- (a) measuring the optical property of the at least one microwell after the at least one cell, the indicator substance, and the nutrient have been loaded and before incubation;
- (b) measuring the optical property of the at least one microwell after incubation; and
- (c) comparing the measured optical property before incubation and after incubation.

14. A method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, the method comprising:

loading into at least one microwell of the array of microwells: (a) at least one cell from the sample; (b) an indicator substance which comprises a pH sensitive dye; and (c) an amount of a nutrient;

applying a membrane to the microfabricated device to retain the at least one cell in the at least one microwell;

incubating the microfabricated device at predetermined conditions for a duration of time to grow a plurality of cells from the at least one cell in the at least one microwell; and

determining a presence or absence of at least one biological entity of interest that has produced an acid in the at least one microwell during incubation based on a change of the indicator substance after incubation.

15. A method of screening for at least one biological entity of interest in a sample using a microfabricated device having a top surface defining an array of microwells, wherein each of the microwells has a bottom, the method comprising:

depositing an indicator substance which comprises a pH sensitive dye into each of a plurality of microwells of the array of microwells;

loading the sample onto the microfabricated device such that at least one microwell of the plurality of microwells receives at least one cell from the sample and an amount of a nutrient;

applying a membrane to the microfabricated device to retain the received at least one cell

and the nutrient in each of the plurality of microwells;

incubating the microfabricated device at predetermined conditions for a duration of time;
and

determining a presence or absence of at least one biological entity of interest that has produced an acid in the at least one microwell of the plurality of microwells during incubation based on a measurement of an optical property of the at least one microwell after incubation, wherein the optical property is influenced by the acid.