© 2021. Published by The Company of Biologists Ltd | Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Imaging host–pathogen interactions using epithelial and bacterial cell infection models

Amber J. Park[1,*], Madison A. Wright[1,*], Elyse J. Roach[1,2] and Cezar M. Khursigara[1,2,‡]

ABSTRACT

The age-old saying, seeing is believing, could not be truer when we think about the value of imaging interactions between epithelial cells and bacterial pathogens. Imaging and culturing techniques have vastly improved over the years, and the breadth and depth of these methods is ever increasing. These technical advances have benefited researchers greatly; however, due to the large number of potential model systems and microscopy techniques to choose from, it can be overwhelming to select the most appropriate tools for your research question. This Review discusses a variety of available epithelial culturing methods and quality control experiments that can be performed, and outlines various options commonly used to fluorescently label bacterial and mammalian cell components. Both light- and electron-microscopy techniques are reviewed, with descriptions of both technical aspects and common applications. Several examples of imaging bacterial pathogens and their interactions with epithelial cells are discussed to provide researchers with an idea of the types of biological questions that can be successfully answered by using microscopy.

KEY WORDS: Microscopy, Tissue culture, Bacteria, Pathogens, Epithelial cells, Co-culture

Introduction

Bacterial pathogens cause a myriad of diseases and interact with host cells in a variety of ways. They may colonize extracellular niches, attach to epithelial cell surfaces, breach host barriers, establish intracellular infections or combine several of these approaches during the process of infection (Alberts et al., 2002; Hurwitz et al., 2015; Ribet and Cossart, 2015). The combination of tissue culture with microscopy is well suited to recapitulate and document these complex microenvironments, and is an excellent tool for the *in vitro* study of host–pathogen interactions. Importantly, experiments aimed at capturing high-resolution images of host–pathogen interactions involve the integration of multiple disciplines and techniques. This Review highlights the cellular systems, and imaging tools and techniques that allow researchers to better understand the biology of bacterial infection, as well as potential targets for disease mitigation. The scope of the article includes studies focusing on cell culture models of human epithelial barriers and bacteria relevant to disease and pathology. Although critical for some research questions, we do not discuss animal models, tissue explants or cellular systems generated using epithelial–immune cell co-cultures (Gordon et al., 2019; Grivel and Margolis, 2009; Jelicks et al., 2013; Leevy et al., 2007; Papazian et al., 2016). Our discussion is organized around two main technical issues – choosing the biological components of the experimental system and assessing which microscopy-based tools are well-suited to label and image various cell compartments or biological processes. A variety of imaging techniques will be addressed, including both light- and electron-microscopy methods, which cover the spatial scale and resolution required to visualize entire cellular barriers down to the cellular ultrastructure (Fig. 1). The discussion will be complemented with examples of recent studies that have used these methods to generate highly informative micrographs that provide data relevant to researchers studying a wide range of subjects related to host-pathogen interactions.

Building the foundation – choosing model systems and quality controls

Selecting and establishing an appropriate experimental model system is an important step, and an often-challenging initial hurdle, when designing an imaging study that involves the host–pathogen interface. Although there is no single *in vitro* model that can be considered a gold standard, there are approaches that might be more relevant depending on the specific research question. Therefore, the selection of epithelial cell type, the culture technique and quality control measures often requires a great deal of consideration before embarking on an imaging project. A selection of common options for building an infection model (e.g. epithelial cell type and culture technique), including relevant advantages and disadvantages of each, is presented in Table 1. The discussion below will focus on the quality control measures that can be used to assess the selected model system.

For any epithelial and bacterial cell infection model, the experiments typically require a balance between having a sufficient population of bacteria to study and not sacrificing the viability and/or integrity of the host model. Cellular viability and toxicity are commonly measured to assess potential cell damage caused by the exposure to the pathogen, and various methods to evaluate these parameters have been reviewed elsewhere (Stoddart, 2011). These readouts can be a good way to titrate parameters of the infection that may, in excess, inordinately affect host cell health to a point where the model is no longer representative of an *in vivo* barrier. For example, increasing the number of bacteria being added to the infection model relative to the number of host cells (i.e. increasing the multiplicity of infection) can increase epithelial cell cytotoxicity (Hertle and Schwarz, 2004). Barrier integrity can be assessed by measuring the transepithelial electrical resistance (TEER) across the cell layer(s) (Srinivasan et al., 2015). Higher TEER measurements are indicative of a confluent epithelial layer with high barrier integrity; conversely, lower TEER measurements signal breaks in the epithelial barrier, resulting from holes or weak cell–cell junctions (Srinivasan et al., 2015). Importantly, average or

[1]Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. [2]Molecular and Cellular Imaging Facility, University of Guelph, Guelph, Ontario N1G 2W1, Canada.
*These authors contributed equally to this work

[‡]Author for correspondence (ckhursig@uoguelph.ca)

C.M.K., 0000-0001-9263-7604

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

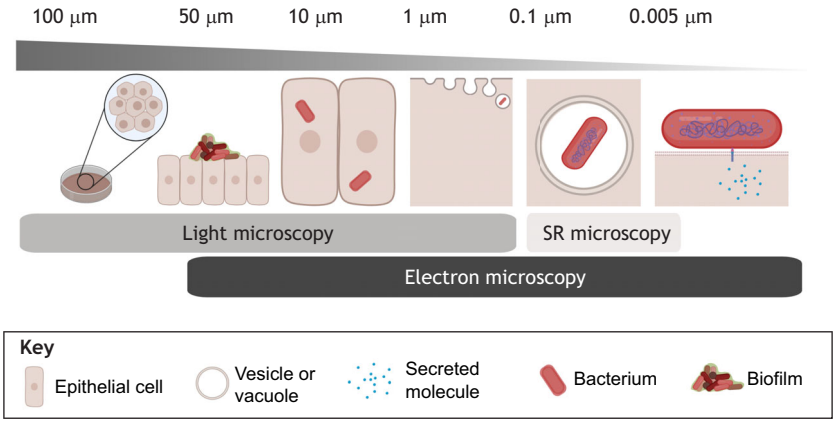

Fig. 1. Biology through a lens. The range of microscopic techniques employed to visualize epithelial cells, *in vitro* infection models and host–pathogen interactions are depicted on a biological scale. Lower resolution light microscopy techniques, such as brightfield, phase contract and differential interference contrast (DIC) microscopy offer general assessment of epithelial cell layer integrity and cell differentiation (100 µm–10 µm). Light-microscopy techniques with higher resolution, including epifluorescence microscopy and confocal laser scanning microscopy (CLSM), allow the imaging of the colonization of epithelial cell layers by bacterial cells, the bacterial internalization process and the subcellular localization of bacterial cells within epithelial cells (100 µm–1 µm). More precise colocalization studies can be performed reliably using super-resolution (SR) microscopy, in which the pattern and architecture formed by specific proteins can be tracked (10 µm–0.1 µm). Electron microscopy can be used to gain insights into protein and effector (e.g. toxins, transcriptional regulators and immuno-regulatory compounds) localization within host subcellular compartments and allows a closer look at host–pathogen interactions (50 µm–0.005 µm).

optimal TEER values can be cell line specific due to variability in the junctional complexes (Srinivasan et al., 2015). The integrity of the epithelial cell barrier can also be assessed by staining adherens junction proteins, such as E-cadherin and β-catenin, and tight junctional proteins, such as zonula occludens-1 (ZO-1, also known as TJP1), claudin-1 and occludin (Barrila et al., 2017; Buckley et al., 2018; Carvalho et al., 2005; Uotani et al., 2019). While viability, toxicity, TEER and junctional protein staining can be determined for all epithelial cell types, cell-type-specific experiments may also be used to validate your model, such as labelling markers of differentiation (Fig. 2A; Table S1). Finally, it is important to note that some experimental conditions, such as prolonged infection or use of highly virulent strains, may require additional or more frequent quality control assessments to ensure the viability and integrity of the infection model is maintained throughout the experiment (Anderson et al., 2008; Starner et al., 2006; Walker et al., 2017). The efforts taken to develop, optimize and evaluate infection models will ensure that an appropriate, biologically relevant system is selected for further microscopic investigation.

Producing a micrograph that is worth a thousand words – imaging tools and techniques

Microscopic techniques can be separated into two main categories: light microscopy and electron microscopy. A description of traditional light and electron microscopy techniques and general uses in host–pathogen imaging studies is provided in Table 2. When choosing the imaging tools and techniques for a host–pathogen interaction experiment, it is important to consider the spatial scale and resolution that will be required to answer the question posed (Fig. 1), and whether you will be working with fixed or live cells. Light microscopy allows both live-cell and fixed-cell imaging but is limited in both lateral (200 nm) and axial (500 nm) resolution due to the relatively long wavelength of visible light (Abbe, 1873; Rayleigh, 1896). Fluorescence capabilities are acquired with the addition of a broad-spectrum light source [i.e. visible and ultraviolet (UV) wavelengths], a series of filters that can fine-tune the source (excitation) and gathered (emission) light, depending on the target and a detector (Young, 1961). Live-cell imaging is less prone to preparation artefacts and can provide important information regarding cellular structures and processes in real-time, but is not compatible with multiple labelling techniques, as described below. Fixing your cells will prevent sample degradation, but the process can produce artefacts, such as the loss of antigenicity and changes to cell morphology (Hobro and Smith, 2017; Jamur and Oliver, 2010a). Compared to light microscopy, electron microscopy provides higher resolution by illuminating the sample with an electron beam, instead of photons, and because of this, it is used to investigate cellular ultrastructure (Gordon, 2014). Samples are fixed, chemically or environmentally, and treated with heavy metal coatings and stains for contrast, which excludes live-cell imaging and prevents the depth of field offered by some light microscopy methods (Winey et al., 2014).

Recently, a group of fluorescence microscopy methods have been developed that challenged the resolution limits of traditional light microscopy. In 2014, Betzig, Hell and Moerner were awarded the Nobel Prize in chemistry for their work in developing super resolved fluorescence microscopies. This group of methods, collectively termed super-resolution microscopy, uses specialized equipment or modifications to typical microscope arrangements, along with extensive post-acquisition image analysis and transformation, which results in resolution beyond the diffraction limit of visible light (Galbraith and Galbraith, 2011; Schermelleh et al., 2019). This means that cellular structures and processes can be seen at a resolution closer to that provided by electron microscopy, while still retaining the benefits of light microscopy, such as live-cell imaging and tools for specific labelling. Some of these super-resolution methods use physical grating, that is, controlled separation of light, to allow for non-uniform delivery of the excitation light source to improve resolution [e.g. structured illumination microscopy (SIM) (Langhorst et al., 2009)]. Alternately, dual objectives [e.g. dual objective multifocal plane microscopy (dMUM) (Ram et al., 2009)], or selective activation of fluorophores can be used [e.g, photo-activated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM) (Henriques et al., 2011)]. A recent review (Vangindertael et al., 2018) discusses the theory behind the various super-resolution techniques in detail and addresses

Table 1. Commonly used options for building an infection model

Option	Description	Advantages	Disadvantages
Cell type			
Immortalized cell lines	Cells that have undergone spontaneous transformation (e.g. cancerous cells) (Hopfer et al., 1996) Cells transformed with inducible oncoproteins to inactivate tumor suppressor genes (Hopfer et al., 1996)	Good reproducibility (Gazdar et al., 2010; Kaur and Dufour, 2012) Easy to use (Kaur and Dufour, 2012) Can undergo unlimited population doublings, creating a large supply (Kaur and Dufour, 2012)	Phenotypic and genotypic changes over time (e.g. genetic drift) (Gazdar et al., 2010; Kaur and Dufour, 2012) Limited differentiation into multiple cell types, which may underrepresent some aspects of cell function (Keller et al., 2010; McCance et al., 1988)
Primary cells	Cells harvested from host tissues, which undergo limited manipulation and are used until they cease to divide (i.e. reach senescence) (Hayflick and Moorhead, 1961)	More representative of epithelial cells in their native tissues (Gazdar et al., 2010; Pohl et al., 2009) May be more resistant to damage (Guevara et al., 2016; Rajan et al., 2000) and bacterial internalization (Kortebi et al., 2017) May more accurately recapitulate clinical features of disease (e.g. changes to barrier integrity) (Uotani et al., 2019) Can differentiate into multiple tissue-specific subtypes (Yonker et al., 2017)	Finite lifespan (Freshney, 2010; Hayflick and Moorhead, 1961) Low availability (Haycock, 2011) High cost (www.atcc.org) Greater interindividual variability (Gazdar et al., 2010); however, if the proper controls are used, can be a better representation of biological variability from patient-to-patient
Immortalized primary cells	Cells generated, without the use of viral oncoproteins, by reducing processes that normally result in the onset of senescence in normal cells (e.g. progressively shortening of telomeres) (Lee et al., 2004; Liu et al., 2010; Ramirez et al., 2004)	Near normal karyotypes (Liu et al., 2010; Ramirez et al., 2004) Gene expression more closely resembles parental cells when compared to cells immortalized with viral oncoproteins (Ramirez et al., 2004) Extended lifespan compared to primary cells (Lee et al., 2004; Liu et al., 2010; Ramirez et al., 2004)	Typically more expensive than immortalized cell lines but less expensive than primary cells (www.atcc.org) May require addition of growth factors to culture medium (Rambhatla et al., 2002)
Stem cells	Cells that have the ability to differentiate into many types of cells and have the ability to self-renew (Daley, 2015)	Potentially large source of stem cells (Haycock, 2011) Can allow expansion of cells for more than 1 year (Bartfeld et al., 2015) Differentiation into multiple cell types (Bartfeld et al., 2015; Daley, 2015)	Difficulties directing differentiation towards a specific phenotype (Haycock, 2011) Unequal differentiation of cells (Haycock, 2011)
Technique: monolayer			
Petri dishes	Cells growing as monolayers directly attached to the bottom of petri dishes (Freshney, 2010) Grown with a covering of media (i.e. submerged cultures) (Freshney, 2010)	Simplest system used for culturing monolayers of epithelial cells (Freshney, 2010) Easily accessible growth surface (Freshney, 2010) Typically an economical option (Freshney, 2010) Easy observation on inverted microscope (Freshney, 2010)	May lack some physiological cues required for epithelial cell polarization and differentiation (Antoni et al., 2015; Barrila et al., 2010; Koledova, 2017)
Multiwell plates	Cells growing as monolayers directly attached to the bottom of multiwell plates (Freshney, 2010) Grown with a covering of media (i.e. submerged cultures) (Freshney, 2010) Cells can also be grown on coverslips placed inside multiwell plates to aid with sample processing (e.g. fixation) (Krokowski et al., 2018)	Amenable to high-throughput methods (Aubey et al., 2019; Ernstsen et al., 2017) Easy observation on inverted microscope (Freshney, 2010)	May lack some physiological cues required for epithelial cell polarization and differentiation (Antoni et al., 2015; Barrila et al., 2010; Koledova, 2017) Size of meniscus compared to well size can make phase contrast microscopy challenging (Freshney, 2010)
Microfluidic devices	Allows submerged cultures to be grown in a specialized flow-chamber that can be connected to a peristaltic pump to apply shear stress during the growth and/or infection stages of the experiment (Pedersen et al., 2018; Sullivan and Ulett, 2018)	Cultures can be fixed (Pedersen et al., 2018) or maintained as live-cell cultures (Pedersen et al., 2018; Sullivan and Ulett, 2018) during infection and/or imaging Allows investigators to incorporate certain physiological cues (Pedersen et al., 2018; Sullivan and Ulett, 2018)	High flow rates usually require large amounts of medium if continuous flow is used (Pedersen et al., 2018) Different types of cells can have different tolerance levels for flow (i.e. some cells are more fragile to flow conditions and others are more flow resistant) (Pedersen et al., 2018)
Permeable inserts	Cells grown on permeable synthetic membranes, often coated with extracellular matrix components (e.g. collagen), are supported by plastic inserts that suspend the membrane within the well of a multiwell plate (Freshney, 2010; Karp et al., 2002)	Can produce cultures that are submerged or have an air-liquid interface by controlling which compartments (apical or basal) contain medium (Karp et al., 2002; Sonoda et al., 2009) and can restrict pathogen exposure to the apical compartment only to mimic biologically relevant conditions (Barrila et al., 2010)	Require longer times to establish (Sonoda et al., 2009) Static culture (Kang and Kim, 2016) While the membranes can be excised from the inserts for imaging when needed (Di Paola et al., 2017), the process is not amenable with high-throughput microscopy

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

Table 1. Continued

Option	Description	Advantages	Disadvantages
Permeable inserts (continued)		Aid with establishing cell polarity (Barrila et al., 2010; Freshney, 2010; Paz et al., 2014; Sonoda et al., 2009) and measuring barrier integrity (Karp et al., 2002; Srinivasan et al., 2015; Starner et al., 2006; Walker et al., 2017) Often able to withstand long periods of exposure to pathogenic bacteria while maintaining integrity of the cell layer (Starner et al., 2006; Walker et al., 2017)	
Techniques: multilayer			
Biological scaffolds	Cells grown in a three-dimensional arrangement on scaffolds, which are typically made up of collagen, extracellular matrix components, and/or synthetic or semi-synthetic materials (Barrila et al., 2010; Haycock, 2011; Ravi et al., 2015) Examples: culturing epithelial cells on top of extracellular matrix material in rotating wall vessel bioreactors (Barrila et al., 2017; Carvalho et al., 2005) or seeding fibroblasts onto scaffolds and then coating with collagen to support the growth of the epithelial cells of interest (Marrazzo et al., 2016)	Support the growth of epithelial cells (Freshney, 2010; Haycock, 2011) Use of fibroblasts or extracellular matrix components can act as stimulus for differentiation (Pohl et al., 2009; Steinke et al., 2014) Inexpensive (Barrila et al., 2010) Resembles the three-dimensional structure of the tissue being studied (Barrila et al., 2010; Haycock, 2011; Ravi et al., 2015)	Requires advanced culturing techniques (Antoni et al., 2015; Barrila et al., 2010) Increased sample thickness can be more challenging to image (Haycock, 2011; Shamir and Ewald, 2014) Scaffolding may restrict some pathogens' access to the host cells (Barrila et al., 2010) May have limited reproducibility between scaffolds (Haycock, 2011)
Spheroids	Three-dimensional aggregates of cells formed by adherent cells sticking to each other without the need of a scaffold (Antoni et al., 2015; Haycock, 2011) Can be made from a variety of cell types (Antoni et al., 2015; Haycock, 2011) Use of non-adherent plates can promote cell-cell attachment (Koledova, 2017)	Can be maintained in culture longer than two-dimensional cultures (Antoni et al., 2015)	Requires advanced culturing techniques and/or culturing equipment (Antoni et al., 2015; Barrila et al., 2010; Marrero et al., 2009) Increased sample thickness can be more challenging to image (Haycock, 2011; Shamir and Ewald, 2014)
Microfluidic chips (also known as organ-on-a-chip)	Chips that contain microchannels, which are lined with living cells and is continuously perfused (Huh et al., 2013; Koledova, 2017) Designed to mimic functional units of organs (Huh et al., 2013)	Can be fabricated to mimic complex tissue microenvironments, tissue and organ level functions, and their physiology (Huh et al., 2013; Koledova, 2017) Conditions can be used that produce highly differentiated cultures for infection, such as application of peristalsis-like mechanical forces or physiological flow and cyclic suction to resemble breathing (Huh et al., 2013; Kim et al., 2016) Can be high throughput (Huh et al., 2013; Koledova, 2017; Srinivasan et al., 2015)	Requires advanced culturing techniques and/or culturing equipment (Antoni et al., 2015; Barrila et al., 2010; Huh et al., 2013) Increased sample thickness can be more challenging to image (Haycock, 2011; Shamir and Ewald, 2014) Challenging to access and harvest the sample inside the microdevice, which prevents some types of imaging (e.g. electron microscopy) (Huh et al., 2013) Total cell counts can be limited by size of culture chamber (Huh et al., 2013) Continuous perfusion can dilute samples (Huh et al., 2013)
Organoids	Three-dimensional models of cells that resemble the structure and/or function of the organ or tissue (Bissell, 2017) Can be made up of pluripotent stem cells or multipotent organ-specific adult stem cells (Kretzschmar and Clevers, 2016) Self-assemble with the addition of extracellular matrix components, such as extracellular matrix-based hydrogels (e.g. Matrigel) or collagen (Bartfeld et al., 2015; Bissell, 2017; Sato and Clevers, 2015)	Cytokeratin staining, cell arrangement (thickness and size), junctional characteristics, and microarchitecture was more similar to native tissue than that of monolayer cultures (Carvalho et al., 2005) Can form from single epithelial cells in 7–10 days (Sato and Clevers, 2015) Cultures can be maintained for long periods of time (>1 year) (Bartfeld et al., 2015)	Requires advanced culturing techniques (Antoni et al., 2015; Barrila et al., 2010) Increased sample thickness can be more challenging to image (Haycock, 2011; Shamir and Ewald, 2014)

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

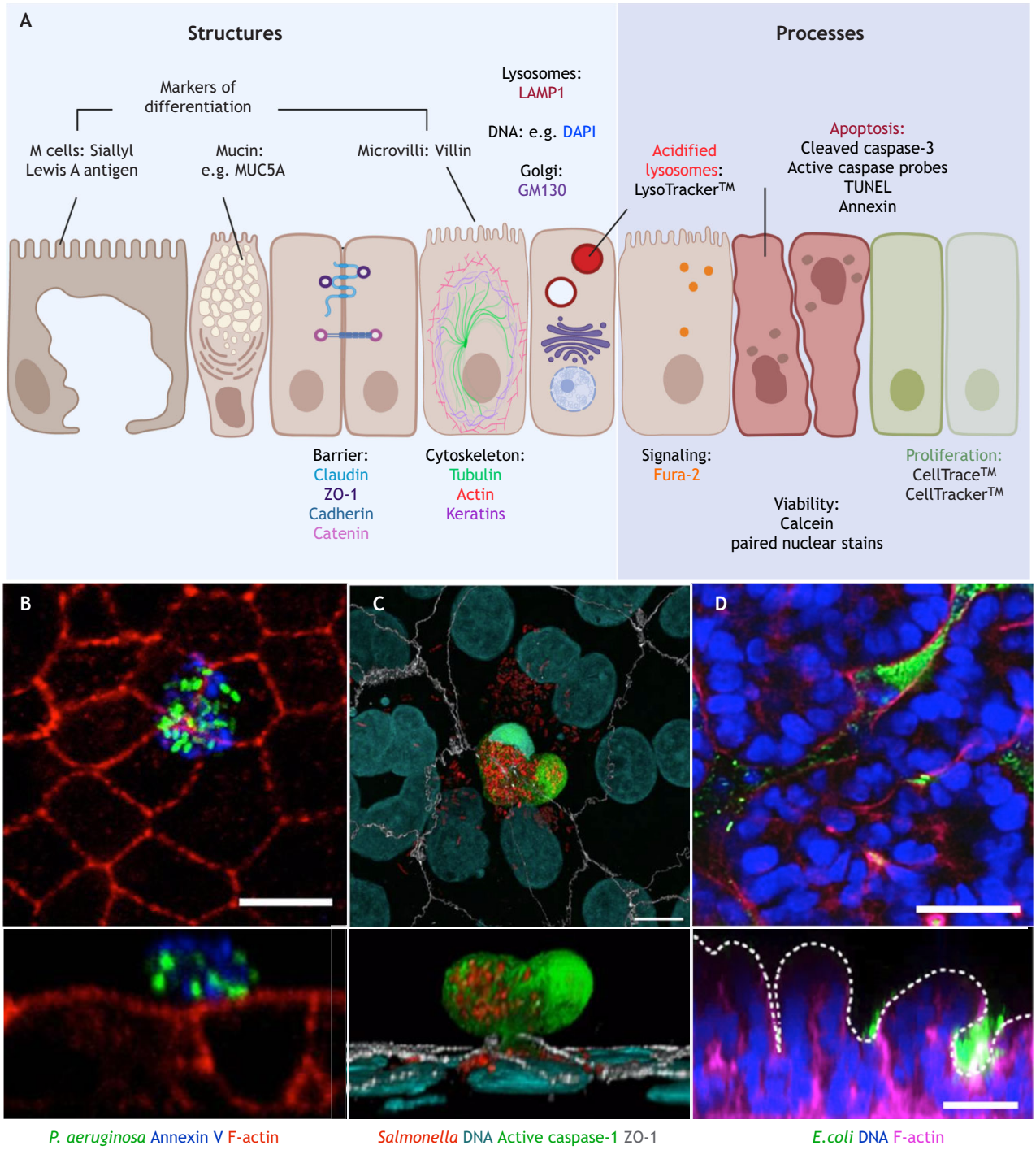

Fig. 2. Tools for labelling structures and processes in host cells. (A) Schematic representation of reagents that can be used to label cellular structures (left) and processes (right) in host cells during infection modelling. A full description and references for these approaches can be found in Table S1. The figure was created with BioRender.com. (B–D) Examples of micrographs demonstrating the use of fluorescent labelling to provide information about cellular responses during infection. (B) Imaging of the cytoskeletal protein F-actin that delineates host cell borders, while Annexin-V labels apoptotic events in a *P. aeruginosa* infection model. These micrographs demonstrate that the bacteria preferentially bind to cell–cell junctions where apoptosis is occurring. Reproduced from Capasso et al. (2016) where it was published under a CC-BY 4.0 license. (C) Imaging of ZO-1 to assess barrier integrity and the inflammatory enzyme caspase-1 show active host cell extrusion and programmed cell death in response to *Salmonella* infection. Reproduced with permission from Knodler et al. (2010). (D) Imaging of F-actin and host-cell DNA were used to visualize the complex architecture of host cells within the 'gut-on-a-chip' infection model, and the location of GFP-labelled *E. coli* microcolonies that preferentially congregated in the intestinal crypt-like structures. Reproduced with permission from Kim et al. (2016). TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling. Scale bars: 10 µm (B,C); 50 µm (D).

their potential use in life sciences. While not specifically focused on infection models, others have described how the biological questions can determine the selection of the specific super-resolution technique (Schermelleh et al., 2019). A striking example of the use of super-resolution microscopy in host–pathogen interactions is the imaging of host cytoskeletal proteins in a *Shigella* infection model (Krokowski

Table 2. Description and potential uses of common light and electron microscopes

Technique	Description	General uses for studying host–pathogen interactions
Brightfield	Simplest form of light microscopy Can image live or fixed samples Relies on inherent contrast Unable to penetrate thick samples No aperture	Gives spatial context Identify tissue-specific structures (e.g. microvilli) Quality control (e.g. to visualize barrier integrity of monolayers, confirm the presence of differentiated cell types)
Phase contrast	Phase ring enhances inherent contrast Can image live or fixed samples Unable to penetrate thick samples No aperture	As for brightfield
Differential interference contrast (DIC)	Combines light from multiple focal planes using a prism Enhanced inherent contrast Yields a pseudo-three-dimensional image Can image live or fixed cells Unable to penetrate thick samples No aperture	As for brightfield
Epifluorescence illumination	Uses reflected fluorescent light High intensity filtered light excites fluorophores Can image live or fixed cells Can penetrate through thicker samples (~10 µm) No aperture Can be combined with computational deconvolution to improve spatial resolution	Image fluorescently labelled surface structures (host or bacterial) Fluorescently labelled bacteria in relation to labelled components of the host cell Images can be overlaid with other micrographs (e.g. brightfield, phase contrast or DIC) to provide context
Confocal laser scanning microscopy (CLSM)	Uses lasers to excite fluorophores Can image live or fixed cells Can penetrate much thicker samples (~100 µm) Uses an aperture to image at a single focal point Laser scans a single focal plane at a time	As for epifluorescence illumination Additional control of the depth of field to image host structures or fluorescently labelled bacteria inside host cells
Transmission electron microscopy (TEM)	Uses electrons to image samples Image is a negative projection from penetrating electrons Samples must be fixed/stained Cannot image through thick samples Uses apertures to image at a single focal plane Can incorporate specific labels For host–pathogen interactions, samples typically prepared by ultra-thin sectioning	Image inside host cells Mechanisms of bacterial entry and intracellular persistence Host ultrastructure (e.g. mucous granules, cilia)
Scanning electron microscopy (SEM)	Uses electrons to image samples Image is a surface scan from backscattered electron Sample thickness does not matter Aperture used to focus electrons in a single focal plane	Bacteria interacting with host cell surfaces Bacterial aggregation (i.e. biofilm) on the surface of epithelial cells Quality control (e.g. to visualize barrier integrity of monolayers, confirm the presence of differentiated cell types)

et al., 2018; Mostowy et al., 2010). In the 2010 paper, the authors used mostly confocal microscopy to show the interaction of host cell cytoskeletal proteins called septins with intracellular bacteria, but also used STORM to capture high-resolution image of fluorescently labelled septin assemblies they refer to as 'cages' surrounding individual *Shigella* (Mostowy et al., 2010). In addition, they also provide a direct comparison between the resolving power of conventional and STORM methods (Mostowy et al., 2010). In subsequent work from the same group, the authors used fluorescent labelling and SIM to capture the overlap of the bacterial division protein filamentation temperature-sensitive protein Z (FtsZ) with septin 7 (SEPT7), which led them to conclude that the microscale change in membrane curvature that occurs during bacterial division can act as a trigger for host cytoskeletal arrangements that result in the entrapment of intracellular bacteria (Krokowski et al., 2018). Others have used STORM to visualize the nanoscale distribution and linear organization of the host ubiquitin on the surface of intracellular *Salmonella* Typhimurium (Van Wijk et al., 2017). These observations, which would not have been possible using traditional light microscopy, contributed to the hypothesis that specific ubiquitylation patterns, or 'signatures', may elicit specific host signalling pathways involved in bacterial clearance (Van Wijk et al., 2017). The main drawbacks to these methods, in general, is the cost and access to specialized equipment and/or software, and the high level of expertise required to use them. Accordingly, these highly technical and challenging methods have not been widely used in the studies that fall within the scope of this Review, but will likely become more prominent in the field in years to come as they become more accessible outside of core facilities or specialized research groups.

Light microscopy imaging tools and techniques

There are many imaging tools that can be used to enhance the power and utility of light microscopy, and the information gained from labelling experiments often provides key clues to pathogenic mechanisms at the host–pathogen interface. Labelling techniques used with light microscopy can be

broadly separated into those that can be used with fixed cells and those that can be used with live cells.

Fixed-cell techniques

Methods used with non-living fixed cells commonly exploit fluorescently labelled antibodies to mark epithelial or bacterial cells, or use natural or synthetic compounds that bind specific cell structures (e.g. nuclei) or intracellular compounds (e.g. Ca^{2+}) (Fig. 2A; Table S1). Antibody-based methods can be completed with traditional multidomain antibodies, or more recently developed single domain alternatives called nanobodies. Nanobodies are a unique form of immunoglobulin G (IgG) isolated from the sera of *Camelidae*, comprising a single heavy chain and a single variable domain, which favourably affects the size, solubility and affinity of nanobodies versus multidomain antibodies (Beghein and Gettemans, 2017), and reduces some labelling artefacts associated with larger multidomain antibodies (Beghein and Gettemans, 2017; Sograte-Idrissi et al., 2020). While nanobodies are not as widely available, or commonly used in traditional light microscopy, they are well suited for high- and super-resolution applications because of their spatial resolution in the nanometer range (Ries et al., 2012), as reviewed recently (Beghein and Gettemans, 2017). Fixed-cell methods can usually be completed without significant technical development prior to imaging and follow similar protocols in bacterial and host cells. A common method to label fixed cells is immunolabelling. These experiments use fluorescently labelled antibodies to detect proteins, and generally follow a standard procedure of fixation, permeabilization, blocking, and addition of primary and/or secondary antibodies that recognize the target antigen (Bratthauer, 2010). The need for a permeabilization step that allows antibodies to cross fixed membranes, and the reagent used to perform this step, will depend on the subcellular location of the antigen (Jamur and Oliver, 2010b) but can also be used as a method of examining specific host–pathogen interactions. For example, selective permeabilization with digitonin, which permeabilizes the plasma membrane but not intracellular membrane-bound organelles, has been used to determine whether intracellular *Salmonella* localize in the cytosol or inside a vacuole during infection of mucosal epithelial cells (Knodler et al., 2010).

With immunolabelling, primary antibodies can recognize a conserved antigen within the target and thus can be used across multiple cell lines (e.g. cytoskeletal or junctional protein antigens) or bacterial species and/or strains [e.g. highly conserved lipopolysaccharide core antigens (Pollack et al., 1987)]. Alternately, antibodies can be specific to a group of bacteria that share a common surface structure (i.e. serotype) (Aubey et al., 2019; Carvalho et al., 2005), or bind a specific protein such as a bacterial virulence factor (Uotani et al., 2019). Another strategy to detect or image specific cellular structures in fixed cells is epitope tagging; here, genetic constructs are generated that allow for the co-expression of polypeptide protein tags (with commercially available antibodies) and a gene of interest (Fritze and Anderson, 2000). Common tags include hemagglutinin (HA) or the Myc epitope. When fluorescently labelled antibodies are applied using indirect immunolabelling protocols (i.e. both primary and secondary antibodies are used), fluorophore-labelled secondary antibodies can be varied to accommodate different colour combinations in multiplexing experiments that simultaneously image multiple proteins within a single sample. When this approach is used, online tools can help to determine the spectral compatibility of dyes and probes before setting up experiments, including SpectraViewer from Thermo Fisher Scientific (https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html#!/) or Spectra Analyzer from Biolegend (https://www.biolegend.com/en-us/spectra-analyzer). After the application of the antibodies, counterstains that bind to specific structures, such as the nucleus or the plasma membrane, provide contrast and cellular context. However, it is important to keep in mind, when using these reagents in co-culture, that some common fluorescent dyes or stains bind to structures that are found in both host cells and bacterial cells. For example, wheat germ agglutinin (WGA)-conjugated dyes bind sialic acid residues, which are found on the surface of epithelial cells but also in some bacterial membranes (Severi et al., 2007). After the labelling protocols, the samples are then mounted, with the option of using anti-fade mounting media (Ono et al., 2001) to prolong the stability of the fluorescent signal for subsequent imaging.

Fixed-cell samples can also be labelled using fluorescent *in situ* hybridization (FISH), which uses fluorescently labelled oligonucleotide probes that recognize a nucleic acid sequence of interest inside fixed cells (Spector et al., 2005; Liehr, 2017). This approach was used to tag Nontypeable *Haemophilus influenzae* (NTHi) in a co-culture system with primary epithelial cells, using a cyanine dye-labelled probe that recognized NTHi 16S RNA (Walker et al., 2017). Although quick and easy to perform in theory, this method is not as commonly used for imaging of host–pathogen interactions, likely due to a lower abundance of the target compared to protein antigens, as well as the potential technical challenges and time investment required for the optimization of the initial FISH protocol in bacteria, including probe design and sample preparation, as reviewed elsewhere (Frickmann et al., 2017; Moter and Göbel, 2000). The main advantage of this technique compared to antibody-based methods is that it can be used when there are no commercially available antibodies for the target or when the production of custom antibodies is cost prohibitive. A list of published rRNA-targeted probes for bacteria can be found on the open-source repository ProbeBase (https://probebase.csb.univie.ac.at/; Greuter et al., 2016).

Live-cell techniques

Live-cell techniques most often involve the use of fluorescent microscopes (Table 2) and fluorescent protein labels. The most-used fluorescent protein is green fluorescent protein (GFP), which has many variants (see Box 1). After a suitable fluorescent protein has been selected, the corresponding gene can be transiently introduced into live bacteria using an autonomously replicating plasmid (Di Paola et al., 2017; Ernstsen et al., 2017; Knodler et al., 2010; Rajan et al., 2000; Starner et al., 2006; Sullivan and Ulett, 2018) (Fig. 3Aiii,Ciii) through standard transformation protocols (Froger and Hall, 2007; Gonzales et al., 2013). Plasmid-based systems are efficient to work with; they can provide a strong signal due to frequently producing multiple copies per bacterium and can often be used with more than one species. However, they commonly require the addition of antibiotics to the culture medium for plasmid maintenance, which may not be ideal for experiments involving several bacterial strains with variable antimicrobial sensitivity. This issue can be circumvented by empirically determining the time frame of plasmid stability following the removal of the antibiotic from the culture and completing the experiments within that window (Starner et al., 2006).

Alternatively, the gene encoding the fluorescent protein can be integrated into the genome by site-specific recombination. Examples of integration vectors include mini-Tn7 (Barnes et al., 2008; Choi et al., 2005) or mini-CTX (Hoang et al., 2000). Barnes and colleagues used the mini-Tn7 vector to demonstrate the adhesion of GFP-labelled *Pseudomonas aeruginosa* to the host

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

Box 1. Variations of GFP

A commonly used fluorescent protein, GFP, or one of its derivatives, began its rise to notoriety in the imaging world following its appearance on the cover of Science in 1994 as a novel marker for gene expression (Chalfie et al., 1994). Synthetic variants of the GFP molecule, which was originally isolated from the jellyfish *Aequorea victoria* (Morise et al., 1974), were developed by random amino acid substitutions that resulted in enhanced stability and brightness (Cormack et al., 1996; Heim et al., 1994). Members of the GFP family are now commercially available with profiles in the cyan (e.g. ECFP), green (e.g. EGFP), yellow (e.g. EYFP) and red (e.g. dsRED) spectral regions. Importantly, the spectral properties of GFP and its variants can be influenced by changes in culture conditions, including temperature and pH (Campbell and Choy, 2001; Ward et al., 1982), so some variants may be more appropriate than others for different culture conditions. On the pathogen side, this can be particularly relevant if researchers are imaging bacteria in multiple cellular compartments with different pH ranges (e.g. lysosomes compared to cytosol); here, an acid-tolerant (pKa<4.0) (Shinoda et al., 2018) or pH-stable fluorescent protein (Roberts et al., 2016) can reduce variability between subcellular compartments. Another variation of the standard GFP is the split-GFP, which has been used as a tool to examine the spatial distribution of T3SS effector molecules from *Salmonella* in living host cells (Van Engelenburg and Palmer, 2010). The system works by tagging a bacterial effector protein with the terminal portion of GFP (GFP11), which only produces a fluorescent signal when it is complemented by the remaining portion of the GFP (GFP1-10) that is produced by the host cell (Van Engelenburg and Palmer, 2010). Others have used this system to study the accumulation and specific distribution of secreted virulence factors of *L. monocytogenes* and *S. enterica* during the course of infection (Batan et al., 2018; Van Engelenburg and Palmer, 2010; Young et al., 2017).

extracellular matrix protein fibronectin (Barnes et al., 2008). One disadvantage to chromosomally expressed GFP, however, is that the signal is often not as strong due to the lower copy number compared to a plasmid-based approach. Once integrated, the gene can be under the control of (1) a constitutive promoter that provides continual expression, (2) an inducible promotor that provides on–off control, or (3) a gene-specific promoter that can also provide information on the expression of genes that, for example, influence virulence. The latter option was elegantly used to track the downregulation of the *Salmonella* type III secretion system (T3SS) genes and flagellar genes following host-cell internalization, and to show differential activation of individual genes depending on the location of the bacteria inside the cell and the time post-infection (Knodler et al., 2010) (Fig. 3Ciii).

Importantly, when labelling a bacterium with a fluorescent protein certain caveats and controls must be considered. First, a signal from a fluorescent protein may not mean a live bacterium, so alternative methods of accurately assessing viability such as LIVE/DEAD staining and estimating cell colony forming units are necessary. Second, maturation and degradation rates of the fluorescent proteins may vary between bacterial species, growth conditions or culture conditions, and standard curves of relative fluorescence units (RFU) versus time should be completed when the signal is used for quantification or assessment of viability, especially in the case of systems with inducible promoters. Finally, the production of large fluorescent proteins can become a metabolic burden so growth curves should be completed to ensure that the use of fluorescent proteins in live-cell systems is not generating experimental artefacts (e.g. decreased growth or virulence).

For host cells, similar approaches can be employed using expression vectors and/or transfection reagents intended for use in eukaryotic cells and either transient or stable transfection protocols (Colosimo et al., 2000). In general, the choice between transient or stable transfection will depend on the timeline of the experiment and how amenable the cells are to manipulation. For shorter experiments (12–72 h) involving cells growing as an undifferentiated monolayer, transient transfection methods can deliver the genetic information required for fluorescent protein production in a large proportion of cells in the culture and provide a fluorescent signal for few days post transfection, before it is lost owing to replication or degradation (Colosimo et al., 2000). This approach was used recently to label autophagic membranes of *Listeria monocytogenes*-infected cells growing in multiwell plates (Kortebi et al., 2017). The transfected protein was expressed up to 3 days post-infection and micrographs from these experiments aided in demonstrating that the bacteria entered a persistent state and became trapped in lysosome-like vacuoles in a subset of cells (Kortebi et al., 2017). In contrast, infection models that involve well-differentiated polarized cells (Rybakovsky et al., 2019) or cell systems that require weeks to establish, are better suited to stable transfection approach that produces cultures with inheritable fluorescence (Colosimo et al., 2000). Some aspects of the stable transfection protocol may require optimization, such as the method of transfection, the selection reagent, the culture medium or the plasmid concentration, which can become a rate-limiting step in the transfection of different cell types. However, the final product, now considered a new cell line, can be a valuable tool for ongoing research. For instance, stable transfection using a lentiviral vector was used to generate mCherry-expressing human gastric epithelial spheroids (Sebrell et al., 2018) that were employed in *Helicobacter pylori* infection studies (Sebrell et al., 2019). Of note, the non-profit plasmid repository Addgene includes information on both prokaryotic and eukaryotic vectors and contains a fluorescent protein guide (www.addgene.org/fluorescent-proteins/) that can aid in the selection of the appropriate tools. In addition, a small selection of stable human GFP- and/or red fluorescent protein (RFP)-expressing cells are now commercially available for use in situations where the transfection protocols cannot be performed and there is flexibility regarding the cell line that can be used.

In addition to labelling cellular structures, reagents are also available to track processes that are relevant to host–pathogen interactions in live host cells (Fig. 2; Table S1); these include the externalization of phosphatidylserine associated with early stages of apoptosis, assessed for instance by Annexin V staining (Fig. 2B), DNA degradation during late stages of apoptosis by TUNEL staining, the activation of caspase enzymes (e.g. caspase-1) linked to inflammation (Fig. 2B), fluctuations in Ca^{2+} that indicate signalling events (by using Fura-2) (Bain et al., 1998), the acidification of lysosomes (e.g. LysoTracker™) (Capasso et al., 2016), as well as cell location or its replication state by using CellTracker™ or CellTrace™ (Capasso et al., 2016). The use of these reagents can provide important information regarding the underlying mechanisms of infection. For example, studies examining the infection of host cell layer with either *P. aeruginosa* (Capasso et al., 2016) (Fig. 2B) or *S.* Typhimurium (Knodler et al., 2010) (Fig. 2C) both showed host-cell extrusion events associated with areas of pathogen accumulation, but the respective use of reagents to track cellular processes suggests distinct underlying mechanisms. In the *P. aeruginosa* infection, Annexin V staining was used to demonstrate that these bacteria preferentially bind to cell–cell junctions in host cells that are undergoing apoptosis (Capasso et al., 2016) (Fig. 2B). Importantly, control experiments showed that under their culture conditions, the bacteria were not inducing host cell death, merely taking advantage

Journal of Cell Science (2021) 134, jcs250647. doi:10.1242/jcs.250647

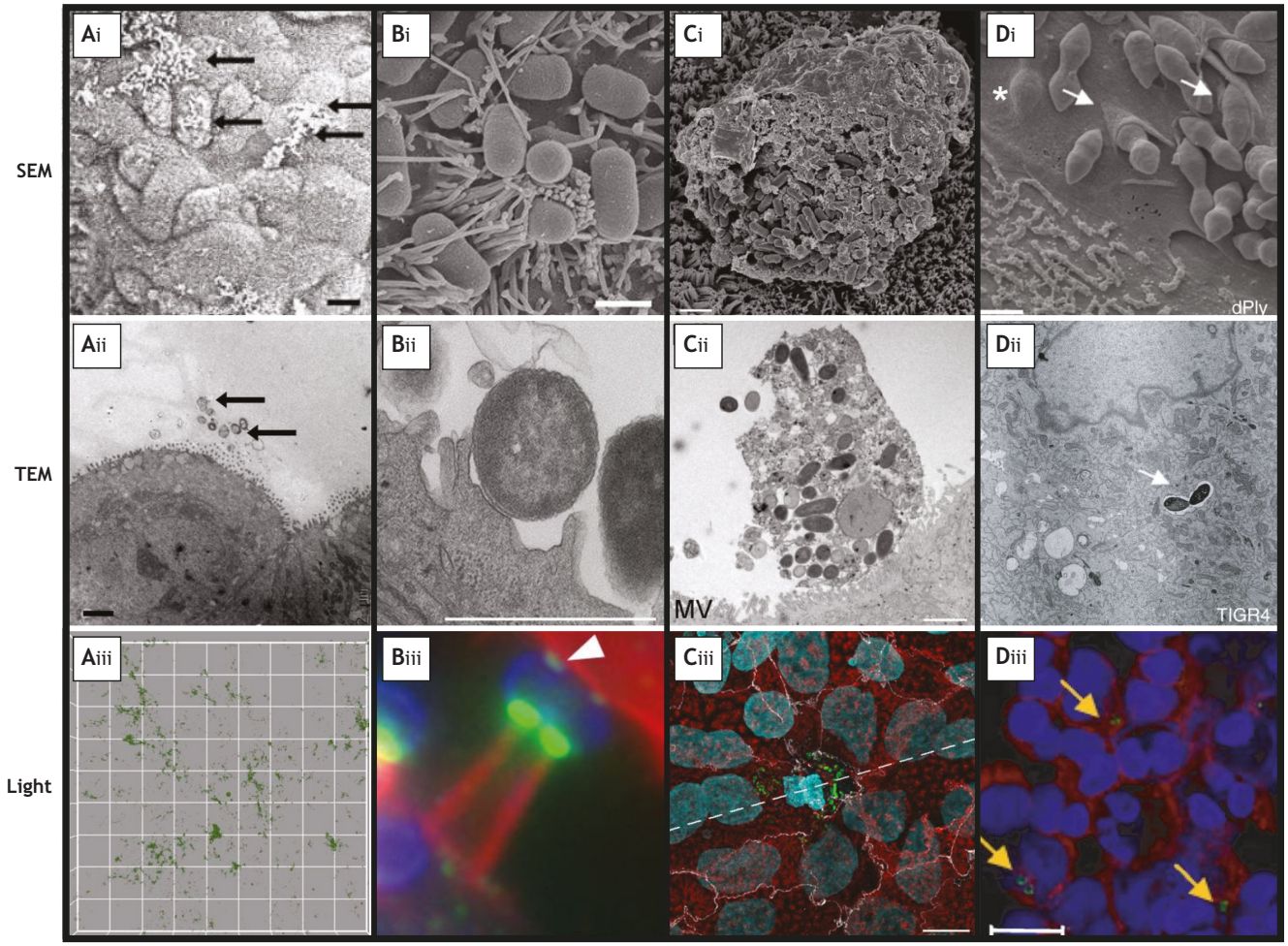

Fig. 3. Complementary views of individual infections models. Here, each column represents a single infection model that has been imaged using SEM (top row), TEM (middle row) and light (bottom row) microscopy. Each imaging technology provides a different type of information, which taken together, can uncover mechanism of attachment (A), dissemination (B,C), or colonization (D) during infection of host cells. The micrographs represent infection models of (Ai–iii) Nontypeable *H. influenzae*, reproduced from Starner et al. (2006) with permission from the American Thoracic Society. (Bi-iii) *Escherichia coli*, reproduced from Velle and Campellone (2017) with where it was published under a CC-BY 4.0 license; (Ci–iii) *Salmonella* Typhimurium, reproduced with permission from Knodler et al. (2010); (Di-iii) *Streptococcus pneumoniae*, reproduced from Weight et al. (2019) where it was published under a CC-BY 4.0 license. The bottom row shows fluorescence imaging of: (Aiii) GFP-labelled NTHi bacteria; (Biii) *E. coli* (blue), epithelial F-actin (red) and bacterial effectors (HA-Tir, green); (Ciii) wild-type *Salmonella* carrying PprgH-GFP[LVA] (green), host cell DNA (cyan), villin (red) and ZO-1 (grey); and (Diii) host nuclei (blue), host surface carbohydrates (WGA, red), and bacteria (specific serotype antiserum, green). Annotations retained from the original papers are not defined here for brevity.

of its outcomes, as indicated by similar rates of apoptosis in uninfected controls (Capasso et al., 2016). In contrast, extrusion events in the *S.* Typhimurium infection model showed that host-cell extrusion was associated with an active inflammatory process involving the increased activation of caspase enzymes, as indicted with caspase-1 immunolabelling (Knodler et al., 2010) (Figs 3Ci–iii and 2C). This type of labelling, therefore, provides information that can be used to target separate and specific host-cell processes when developing anti-infective therapy for these pathogens.

In addition, time-lapse or real-time imaging in host–pathogen interaction is important as it captures the dynamic nature of the interactions during the infection process. Examples include the recording of ciliary beat frequency to determine whether *H. influenzae* exposure affects mechanical innate immune responses (i.e. mucociliary clearance) (Walker et al., 2017). Time-lapse imaging can also be used to visualize intracellular motility of various pathogens to examine the mechanism involved in host-cell transmigration (Golovkine et al., 2016), motility within host cells (Nieto et al., 2019), or the switch between dissemination and persistence (Kortebi et al., 2017; Nieto et al., 2019). It has also been used for the verification of intracellular replication (Lerner et al., 2016; Nieto et al., 2019). Finally, real-time imaging is an excellent technique for visualizing bacterial translocation across the surface of cultured cells, and has been used to demonstrate the cell–cell transmission of pathogenic *Escherichia coli* (Sanger et al., 1996; Velle and Campellone, 2017), which has the important consequence of expanding the range of bacterial colonization within a host.

For all techniques described above that use fluorescence microscopy, it is important to assess non-specific fluorescence (i.e. autofluorescence) that can arise from the cell culture media (e.g. riboflavin) or autofluorescent components that naturally occur in host tissues (e.g. collagen and tryptophan). Here, washing out, modifying or using specific imaging media (e.g. FluoroBrite™) (Di Paola et al., 2017) can help limit autofluorescence, as can the use of blocking and quenching reagents (e.g. animal serum, BSA and TrueBlack® Lipofuscin) during sample processing.

Electron microscopy imaging tools and techniques

Electron microscopy provides greater image resolution compared to light microscopy and, therefore, is commonly used to investigate the ultrastructure of cells and their components. The two main types of electron microscopy are transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Table 2), and both are commonly and effectively used to study host–pathogen interactions.

TEM requires samples to be fixed either chemically, with aldehydes and osmium tetraoxide, or by using an environment with cryogenic temperatures at high pressure, termed high pressure freezing and freeze substitution; afterwards, the samples are embedded in a plastic resin and sliced in thin sections for imaging (Bozzola and Russel, 1998). Importantly, the options at each step may introduce reagent- or method-specific artefacts, such as shrinkage and masking of antigens, which have been discuss extensively elsewhere (Maunsbach and Afzelius, 1999). While the preparation of thin sections is technically challenging, this method has resulted in very detailed and informative images of host–pathogen interactions. For instance, the specific location of *Bordetella pertussis* attachment to cell monolayers and the ultrastructure surrounding the site of host–pathogen interactions have been shown in detail by TEM (Guevara et al., 2016). TEM was an important tool in this study because it offered the resolution to show the interaction of individual bacteria with individual cilia on the highly differentiated epithelial cells (Guevara et al., 2016). From this, the authors were able to discern patterns of intimate attachment, including the clustering of multiple bacteria and the attachment to cilial 'tufts' (Guevara et al., 2016). Furthermore, the 'cross-section' view afforded by thin-sectioning allows for the visualization of areas of adherence, invasion, bacterial replication and host cytoskeletal changes all in the same sample, and thus provides a detailed overview of the infection process in this model (Guevara et al., 2016). One of the most common applications of thin-sectioning and TEM is the visualization of internalized bacteria. For instance, TEM has been used to demonstrate that internalized *P. aeruginosa* reside within an intracellular membrane vesicle (Capasso et al., 2016). Similarly, *Streptococcus pneumoniae* were found to reside within a vacuole after infection, but were also shown to be able to transmigrate between epithelial cells to spread infection (Weight et al., 2019) (Fig. 3Dii). TEM can also be used as a quality control protocol to confirm specific characteristics of an infection model that may be relevant to host–pathogen interactions, such as the presence of mucus granules, where *H. influenza* were shown to congregate during the early stages of infection (Marrazzo et al., 2016). With TEM, specific cellular structures can be labelled by gold-conjugated antibodies, using similar methods as those described above for light-microscopy-based immunolabelling experiments.

SEM can also provide high-resolution ultrastructural information on host–pathogen interactions. Processing is, in general, less laborious than TEM, with the vast majority of samples following a standard protocol of chemical fixation, dehydration by critical point drying and then coating with a thin layer of electrically conductive metal, such as gold (Fischer et al., 2012). Importantly, because SEM generates images by scanning the surface of a sample with electrons rather than penetrating the sample, visualization is limited to the observation of extracellular host–pathogen interactions. Accordingly, SEM is often used to study the initial attachment of bacterial cells on a monolayer. For example, SEM studies were able to detect epithelial membrane folding as a response to *S. pneumoniae* interaction (Weight et al., 2019) (Fig. 3Di). This was an important observation both because *S. pneumoniae* is conventionally considered an extracellular pathogen, and the folds were different from the membrane ruffles previously seen during invasion by intracellular pathogens such as *S.* Typhimurium, suggesting a novel mechanism of invasion (Weight et al., 2019). In addition, SEM is a good tool for studying bacterial biofilms, as it can demonstrate both bacterial cell clustering and production of exopolymeric substances (EPS), both of which are hallmark features of biofilm formation (Flemming and Wingender, 2010). For example, SEM was used to show that *H. influenzae* forms biofilms more readily on the surface of epithelial cells from patients with primary ciliary dyskinesia as compared to healthy controls (Walker et al., 2017). Combined with TEM, SEM was also used to show that *H. influenzae* formed adherent biofilms on the apical surface airway epithelia of patients with cystic fibrosis (Starner et al., 2006) (Fig. 3Ai,ii). It is important to note that unlike light microscopy, and to some degree TEM, specific cell structures or processes cannot be labelled when samples are prepared according to the SEM protocol; however, more innovative techniques are continuing to push the limits of what both TEM and SEM can tell us about host–pathogen interactions.

Looking forward – advanced electron microscopy techniques

Understanding host–pathogen interactions is critical to advancing our knowledge and treatment of bacterial infections. The studies referenced and discussed in this Review provide a small subset of the observations that have been previously made using microscopy, and undoubtedly, with the technological advances in imaging techniques, the research questions that can be answered with microscopy will continue to grow. One of the major limitations with traditional electron microscopy methods is sample thickness. As described above, imaging has been traditionally limited to the surface of intact samples in SEM and to serially sectioned samples in TEM. Recently this limitation has been addressed by techniques that automate the removal of precise layers from the surface of a sample using either an ultramicrotome, in a process known as serial block face (SBF), or a gallium ion source, described as focused ion beam (FIB) milling. Both techniques, and how they contribute to three-dimensional sample imaging, have been reviewed elsewhere (Peddie and Collinson, 2014). These techniques can be used with samples that have been prepared in a variety of ways (e.g. embedded, fixed, dried or cryo-preserved). Samples are alternately milled then imaged, resulting in a *Z*-stack of SEM or TEM images, which combined with post-acquisition image analysis can give rise to high-resolution three-dimensional images. A workflow for FIB processing of infected eukaryotic cells has been published elsewhere (Medeiros et al., 2018), as well as a detailed description of combining FIB with SEM to image tissues and epithelial cells (Drobne, 2013) and a workflow for SBF-SEM (Russell et al., 2017). Although these methods are labour intensive, and require optimization and extensive sample preparation, the use of these techniques with electron microscopy has the potential to generate highly informative images of pathogens interacting with the internal compartments of host cells in three-dimensional space.

Likewise, several research questions in host–pathogen interaction studies require the high magnification and resolution presented by electron microscopy but would also benefit from the specific labelling, spatial resolution and depth of field offered by fluorescence microscopy. In addition to super-resolution techniques discussed above, correlative light electron microscopy (CLEM) (reviewed by de Boer et al., 2015) will likely emerge as another key tool in the future of host–pathogen imaging. While not

extensively used in studies that fall within the scope of this Review, CLEM can, in theory, provide detailed cellular context for structures and processes labelled by fluorescent proteins or be used to pre-select rare events for EM analysis. A recent methods paper describes a workflow that can be used to combine confocal light microscopy of intact epithelial monolayers with SEM to address interactions between polarized epithelial cells and bacterial pathogens (Kommnick et al., 2019). In a study discussed above, CLEM had also been used to obtain information about the ultrastucuture within infected host cells (Mostowy et al., 2010). Specifically, the authors used fluorescence light microscopy to identify the host SEPT6–GFP surrounding intracellular *Shigella*, and then employed SEM to determine the morphology of the filamentous septin assemblies within the host cell at high resolution (Mostowy et al., 2010). Importantly, the authors were able to use SEM to image the ultrastrucuture of the interior of the host cell because they first removed the host-cell membrane by treatment with Triton X-100 in a cytoskeleton stabilization buffer (Mostowy et al., 2010). Immunogold labelling was also used to identify the specific location of host-cell phosphorylated myosin light chain (pMLC) and SEPT6, in the samples prepared for SEM, which supported the conclusion that these specific host proteins form the cages that serve to compartmentalize the intracellular bacterium (Mostowy et al., 2010). In a more recent study, CLEM was used to correlate images of fluorescently labelled *Mycobacterium tuberculosis* and host-cell membranes with SEM images to locate replicating bacteria within the cytosol and autophagosomes of the host cell (Lerner et al., 2016). In that study, SBF-SEM was used to gain access to the inside of the host cell at the precise location of previously identified population of EGFP-labelled *M. tuberculosis* (Lerner et al., 2016). The results from the CLEM experiments showed that a subpopulation of *M. tuberculosis* is able to grow in LC3-positive autophagic compartments within host lymphatic endothelial cells, which are specialized epithelial cells that line lymphatic vessels (Lerner et al., 2016). The authors noted that finding replicating bacteria in this location was unexpected considering the established role of autophagy in the antimycobacterial host immune response (Lerner et al., 2016). Importantly, obtaining this specific observation required the incorporation of live-cell imaging, specific light-microscopy-based labelling, preselection of areas within the host cell that contain bacteria and ultrastructural information about host cell compartments, which could only be accomplished using CLEM techniques.

Conclusions and perspectives

While the imaging approaches and recent technical advances discussed throughout this Review have contributed greatly to the advances in the field, we would also like to see more complexity added at the level of experimental design. This could include studies that use multiple bacterial strains (laboratory strains and/or clinical strains) (Hendricks et al., 2016; Starner et al., 2006; Weight et al., 2019), and/or epithelial cell lines (Weight et al., 2019), types (Guevara et al., 2016; Hendricks et al., 2016), or models (Carvalho et al., 2005), to demonstrate universal mechanisms that are independent of model-specific variables. Furthermore, we chose to focus on imaging studies of two-component models (i.e. one epithelial cell and one pathogen) because the relative simplicity of the cell culture techniques makes the models discussed here accessible to most research laboratories, and because this reductionist approach is an important first step in understanding the basic interactions between bacteria and host epithelial barriers. However, as our understanding of bacterial interspecies communication (Azimi et al., 2020) and community-based bacterial phenotypes (e.g. cheating and competition) (Dunny et al., 2008; Rainey and Rainey, 2003), as well as the collective role of bacterial communities in the expression of infectious disease (Chow et al., 2011; Libertucci and Young, 2019; Vayssier-Taussat et al., 2014) progresses, it will be important to expand these models to include more than one pathogen. Similarly, adding complexity by using host models with more than one mammalian cell type (Barrila et al., 2017; Noel et al., 2017; Papazian et al., 2016) will expand the utility of infection modelling that is based on cell culture systems. Importantly, as the complexity of both imaging and infection modelling continues to increase, collaborative studies between research groups with expertise in each of these areas will become even more important, allowing for the combination of multiple, powerful and complementary methods that will allow scientists to improve their knowledge of host–pathogen interactions.

Acknowledgements

The authors would like thank Dr Michaela Strüder-Kypke, manager of Advanced Light Microscopy Unit within the Molecular and Cellular Imaging Facility, University of Guelph, for her insightful comments and guidance during the writing of this manuscript.

Competing interests

The authors declare no competing or financial interests.

Funding

Our work in this area is supported by operating grants from Canadian Institutes of Health Research (CIHR) awarded to C.M.K. (PJT 156111).

Supplementary information

Supplementary information available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.250647.supplemental

References

Abbe, E. (1873). Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung [Contributions to the theory of the microscope and microscopic observations]. I. Construction of a microscope on the basis of this theory]. *Arch. für mikroskopische Anat.* **9**, 413-418. doi:10.1007/BF02956173

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). Cell biology of infection. In *Molecular Biology of the Cell*, chapter 25. 4th edn. Garland Science.

Anderson, G. G., Moreau-Marquis, S., Stanton, B. A. and O'Toole, G. A. (2008). *In vitro* analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect. Immun.* **76**, 1423-1433. doi:0.1128/IAI.01373-07

Antoni, D., Burckel, H., Josset, E. and Noel, G. (2015). Three-dimensional cell culture: a breakthrough in vivo. *Int. J. Mol. Sci.* **16**, 5517-5527. doi:10.3390/ijms16035517

Aubey, F., Corre, J.-P., Kong, Y., Xu, X., Obino, D., Goussard, S., Lapeyrere, C., Souphron, J., Couturier, C., Renard, S. et al. (2019). Inhibitors of the *Neisseria meningitidis* PilF ATPase provoke type IV pilus disassembly. *Proc. Natl. Acad. Sci. USA* **116**, 8481-8486. doi:10.1073/pnas.1817757116

Azimi, S., Klementiev, A. D., Whiteley, M. and Diggle, S. P. (2020). Bacterial quorum sensing during infection. *Annu. Rev. Microbiol.* **74**, 201-219. doi:10.1146/annurev-micro-032020-093845

Bain, C., Keller, R., Collington, G. K., Trabulsi, L. R. and Knutton, S. (1998). Increased levels of intracellular calcium are not required for the formation of attaching and effacing lesions by enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **66**, 3900-3908. doi:10.1128/IAI.66.8.3900-3908.1998

Barnes, R. J., Leung, K. T., Schraft, H. and Ulanova, M. (2008). Chromosomal *gfp* labelling of *Pseudomonas aeruginosa* using a mini-Tn 7 transposon: application for studies of bacteria–host interactions. *Can. J. Microbiol.* **54**, 48-57. doi:10.1139/W07-118

Barrila, J., Radtke, A. L., Crabbé, A., Sarker, S. F., Herbst-Kralovetz, M. M., Mark Ott, C. and Nickerson, C. A. (2010). Organotypic 3D cell culture models: using the rotating wall vessel to study host–pathogen interactions. *Nat. Rev. Microbiol.* **8**, 791-801. doi:10.1038/nrmicro2423

Barrila, J., Yang, J., Crabbé, A., Sarker, S. F., Liu, Y., Ott, C. M., Nelman-Gonzalez, M. A., Clemett, S. J., Nydam, S. D., Forsyth, R. J. et al. (2017). Three-dimensional organotypic co-culture model of intestinal epithelial cells and

macrophages to study *Salmonella enterica* colonization patterns. *NPJ Microgravity* **3**, 10. doi:10.1038/s41526-017-0011-2
Bartfeld, S., Bayram, T., Van De Wetering, M., Huch, M., Begthel, H., Kujala, P., Vries, R., Peters, P. J. and Clevers, H. (2015). *In vitro* expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology* **148**, 126-136.e6. doi:10.1053/j.gastro.2014.09.042
Batan, D., Braselmann, E., Minson, M., Nguyen, D. M. T., Cossart, P. and Palmer, A. E. (2018). A multicolor split-fluorescent protein approach to visualize *Listeria* protein secretion in infection. *Biophys. J.* **115**, 251-262. doi:10.1016/j.bpj.2018.03.016
Beghein, E. and Gettemans, J. (2017). Nanobody technology: a versatile toolkit for microscopic imaging, protein-protein interaction analysis, and protein function exploration. *Front. Immunol.* **8**, 771. doi:10.3389/fimmu.2017.00771
Bissell, M. J. (2017). Goodbye flat biology - time for the 3rd and the 4th dimensions. *J. Cell Sci.* **130**, 3-5. doi:10.1242/jcs.200550
Bozzola, J. J. and Russel, L. D. (1998). *Electron Microscopy: Principles and Techniques for Biologists*. 2nd edn. Boston, MA: Jones and Bartlett Publishers.
Bratthauer, G. L. (2010). Processing of tissue culture cells. In *Methods in Molecular Biology* (eds Constance Oliver and Maria Célia Jumur), Humana Press. pp. 85-92. doi:10.1007/978-1-59745-324-0_12
Buckley, A. G., Looi, K., Iosifidis, T., Ling, K.-M., Sutanto, E. N., Martinovich, K. M., Kicic-Starcevich, E., Garratt, L. W., Shaw, N. C., Lannigan, F. J. et al. (2018). Visualisation of multiple tight junctional complexes in human airway epithelial cells. *Biol. Proced. Online* **20**, 3. doi:10.1186/s12575-018-0070-0
Campbell, T. N. and Choy, F. Y. M. (2001). The effect of pH on green fluorescent protein: a brief review. *Mol. Biol. Today* **2**, 1-4.
Capasso, D., Pepe, M. V., Rossello, J., Lepanto, P., Arias, P., Salzman, V. and Kierbel, A. (2016). Elimination of *Pseudomonas aeruginosa* through efferocytosis upon binding to apoptotic cells. *PLoS Pathog.* **12**, e1006068. doi:10.1371/journal.ppat.1006068
Carvalho, H. M., Teel, L. D., Goping, G. and O'Brien, A. D. (2005). A three-dimensional tissue culture model for the study of attach and efface lesion formation by enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Cell. Microbiol.* **7**, 1771-1781. doi:10.1111/j.1462-5822.2004.00594.x
Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805. doi:10.1126/science.8303295
Choi, K.-H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. A. R. and Schweizer, H. P. (2005). A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods* **2**, 443-448. doi:10.1038/nmeth765
Chow, J., Tang, H. and Mazmanian, S. K. (2011). Pathobionts of the gastrointestinal microbiota and inflammatory disease. *Curr. Opin. Immunol.* **23**, 473-480. doi:10.1016/j.coi.2011.07.010
Colosimo, A., Goncz, K. K., Holmes, A. R., Kunzelmann, K., Novelli, G., Malones, R. W., Bennett, M. J. and Gruenert, D. C. (2000). Transfer and expression of foreign genes in mammalian cells. *BioTechniques* **29**, 314-331. doi:10.2144/00292rv01
Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33-38. doi:10.1016/0378-1119(95)00685-0
Daley, G. Q. (2015). Stem cells and the evolving notion of cellular identity. *Philos. Trans. R. Soc. B Biol. Sci.* **370**, 20140376. doi:10.1098/rstb.2014.0376
de Boer, P., Hoogenboom, J. P. and Giepmans, B. N. G. (2015). Correlated light and electron microscopy: ultrastructure lights up! *Nat. Methods* **12**, 503-513. doi:10.1038/nmeth.3400
Di Paola, M., Park, A. J., Ahmadi, S., Roach, E. J., Wu, Y.-S., Struder-Kypke, M., Lam, J. S., Bear, C. E. and Khursigara, C. M. (2017). SLC6A14 is a genetic modifier of cystic fibrosis that regulates *Pseudomonas aeruginosa* attachment to human bronchial epithelial cells. *MBio* **8**, e02073-17. doi:10.1128/mBio.02073-17
Drobne, D. (2013). 3D imaging of cells and tissues by focused ion beam/scanning electron microscopy (FIB/SEM). In *Nanoimaging* (eds Alioscka A. Sousa and Michael J. Kruhlak), pp. 275-292. Totowa, NJ: Humana Press.
Dunny, G. M., Brickman, T. J. and Dworkin, M. (2008). Multicellular behavior in bacteria: communication, cooperation, competition and cheating. *BioEssays* **30**, 296-298. doi:10.1002/bies.20740
Ernstsen, C. L., Login, F. H., Jensen, H. H., Nørregaard, R., Møller-Jensen, J. and Nejsum, L. N. (2017). Detection and quantification of intracellular bacterial colonies by automated, high-throughput microscopy. *J. Microbiol. Methods* **139**, 37-44. doi:10.1016/j.mimet.2017.05.001
Fischer, E. R., Hansen, B. T., Nair, V., Hoyt, F. H. and Dorward, D. W. (2012). Scanning electron microscopy. *Curr. Protoc. Microbiol.* **25**, 2B.2.1-2B.2.47. doi:10.1002/9780471729259.mc02b02s25
Flemming, H.-C. and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* **8**, 623-633. doi:10.1038/nrmicro2415
Freshney, R. I. (2010). *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*, 6th edn. Hoboken: John Wiley & Sons, Inc.
Frickmann, H., Zautner, A. E., Moter, A., Kikhney, J., Hagen, R. M., Stender, H. and Poppert, S. (2017). Fluorescence *in situ* hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. *Crit. Rev. Microbiol.* **43**, 263-293. doi:10.3109/1040841X.2016.1169990
Fritze, C. E. and Anderson, T. R. (2000). Epitope tagging: general method for tracking recombinant proteins. *Methods Enzymol.* **327**, 3-16. doi:10.1016/S0076-6879(00)27263-7
Froger, A. and Hall, J. E. (2007). Transformation of plasmid DNA into *E. coli* using the heat shock method. *J. Vis. Exp.* **6**, 253. doi:10.3791/253
Galbraith, C. G. and Galbraith, J. A. (2011). Super-resolution microscopy at a glance. *J. Cell Sci.* **124**, 1607-1611. doi:10.1242/jcs.080085
Gazdar, A. F., Gao, B. and Minna, J. D. (2010). Lung cancer cell lines: useless artifacts or invaluable tools for medical science? *Lung Cancer* **68**, 309-318. doi:10.1016/j.lungcan.2009.12.005
Golovkine, G., Faudry, E., Bouillot, S., Elsen, S., Attrée, I. and Huber, P. (2016). *Pseudomonas aeruginosa* transmigrates at epithelial cell-cell junctions, exploiting sites of cell division and senescent cell extrusion. *PLoS Pathog.* **12**, e1005377. doi:10.1371/journal.ppat.1005377
Gonzales, M. F., Brooks, T., Pukatzki, S. U. and Provenzano, D. (2013). Rapid protocol for preparation of electrocompetent *Escherichia coli* and *Vibrio cholerae*. *J. Vis. Exp.* **80**, 50684. doi:10.3791/50684
Gordon, R. E. (2014). Electron microscopy: a brief history and review of current clinical application. *Methods Mol. Biol.* **1180**, 119-135. doi:10.1007/978-1-4939-1050-2_7
Gordon, O., Ruiz-Bedoya, C. A., Ordonez, A. A., Tucker, E. W. and Jain, S. K. (2019). Molecular imaging: a novel tool to visualize pathogenesis of infections in situ. *MBio* **10**, e00317-19. doi:10.1128/mBio.00317-19
Greuter, D., Loy, A., Horn, M. and Rattei, T. (2016). probeBase—an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Res.* **44**, D586-D589. doi:10.1093/nar/gkv1232
Grivel, J.-C. and Margolis, L. (2009). Use of human tissue explants to study human infectious agents. *Nat. Protoc.* **4**, 256-269. doi:10.1038/nprot.2008.245
Guevara, C., Zhang, C., Gaddy, J. A., Iqbal, J., Guerra, J., Greenberg, D. P., Decker, M. D., Carbonetti, N., Starner, T. D., McCray, P. B. et al. (2016). Highly differentiated human airway epithelial cells: a model to study host cell–parasite interactions in pertussis. *Infect. Dis. (Auckl).* **48**, 177-188. doi:10.3109/23744235.2015.1100323
Haycock, J. W. (2011). 3D cell culture: a review of current approaches and techniques. *Methods Mol. Biol.* **695**, 1-15. doi:10.1007/978-1-60761-984-0_1
Hayflick, L. and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585-621. doi:10.1016/0014-4827(61)90192-6
Heim, R., Prasher, D. C. and Tsien, R. Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **91**, 12501-12504. doi:10.1073/pnas.91.26.12501
Hendricks, M. R., Lashua, L. P., Fischer, D. K., Flitter, B. A., Eichinger, K. M., Durbin, J. E., Sarkar, S. N., Coyne, C. B., Empey, K. M. and Bomberger, J. M. (2016). Respiratory syncytial virus infection enhances *Pseudomonas aeruginosa* biofilm growth through dysregulation of nutritional immunity. *Proc. Natl. Acad. Sci. USA* **113**, 1642-1647. doi:10.1073/pnas.1516979113
Henriques, R., Griffiths, C., Rego, E. H. and Mhlanga, M. M. (2011). PALM and STORM: unlocking live-cell super-resolution. *Biopolymers* **95**, 322-331. doi:10.1002/bip.21586
Hertle, R. and Schwarz, H. (2004). Serratia marcescens internalization and replication in human bladder epithelial cells. *BMC Infect. Dis.* **4**, 16. doi:10.1186/1471-2334-4-16
Hoang, T. T., Kutchma, A. J., Becher, A. and Schweizer, H. P. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59-72. doi:10.1006/plas.1999.1441
Hobro, A. J. and Smith, N. I. (2017). An evaluation of fixation methods: Spatial and compositional cellular changes observed by Raman imaging. *Vib. Spectrosc.* **91**, 31-45. doi:10.1016/j.vibspec.2016.10.012
Hopfer, U., Jacobberger, J. W., Gruenert, D. C., Eckert, R. L., Jat, P. S. and Whitsett, J. A. (1996). Immortalization of epithelial cells. *Am. J. Physiol. Cell Physiol.* **270**, C1-C11. doi:10.1152/ajpcell.1996.270.1.C1
Huh, D., Kim, H. J., Fraser, J. P., Shea, D. E., Khan, M., Bahinski, A., Hamilton, G. A. and Ingber, D. E. (2013). Microfabrication of human organs-on-chips. *Nat. Protoc.* **8**, 2135-2157. doi:10.1038/nprot.2013.137
Hurwitz, J. L., Orihuela, C., DiRita, V. J. and Tuomanen, E. (2015). Bacterial interactions with mucosal epithelial cells. In *Mucosal Immunology* (eds Jiri Mestecky, Warren Strober, Michael W. Russell, Brian L. Kelsall, Hilde Cheroutre and Bart N. Lambrecht), 4th edn. pp. 955-973. Elsevier.
Jamur, M. C. and Oliver, C. (2010a). Cell fixatives for immunostaining. *Methods Mol. Biol.* **588**, 55-61. doi:10.1007/978-1-59745-324-0_8
Jamur, M. C. and Oliver, C. (2010b). Permeabilization of cell membranes. *Methods Mol. Biol.* **588**, 63-66. doi:10.1007/978-1-59745-324-0_9
Jelicks, L. A., Lisanti, M. P., MacHado, F. S., Weiss, L. M., Tanowitz, H. B. and Desruisseaux, M. S. (2013). Imaging of small-animal models of infectious diseases. *Am. J. Pathol.* **182**, 296-304. doi:10.1016/j.ajpath.2012.09.026
Kang, T. and Kim, H. (2016). Farewell to animal testing: innovations on human intestinal microphysiological systems. *Micromachines* **7**, 107. doi:10.3390/mi7070107

Karp, P. H., Moninger, T. O., Weber, S. P., Nesselhauf, T. S., Launspach, J. L., Zabner, J. and Welsh, M. J. (2002). An in vitro model of differentiated human airway epithelia. Methods for establishing primary cultures. *Methods Mol. Med.* **188**, 115-137. doi:10.1385/1-59259-185-X:115

Kaur, G. and Dufour, J. M. (2012). Cell lines: valuable tools or useless artifacts. *Spermatogenesis* **2**, 1-5. doi:10.4161/spmg.19885

Keller, P. J., Lin, A. F., Arendt, L. M., Klebba, I., Jones, A. D., Rudnick, J. A., DiMeo, T. A., Gilmore, H., Jefferson, D. M., Graham, R. A. et al. (2010). Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. *Breast Cancer Res.* **12**, R87. doi:10.1186/bcr2755

Kim, H. J., Li, H., Collins, J. J. and Ingber, D. E. (2016). Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc. Natl. Acad. Sci. USA* **113**, E7-E15. doi:10.1073/pnas.1522193112

Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M. and Steele-Mortimer, O. (2010). Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. USA* **107**, 17733-17738. doi:10.1073/pnas.1006098107

Koledova, Z. (2017). 3D cell culture: an introduction. *Methods Mol. Biol.* **1612**, 1-11. doi:10.1007/978-1-4939-7021-6

Kommnick, C., Lepper, A. and Hensel, M. (2019). Correlative light and scanning electron microscopy (CLSEM) for analysis of bacterial infection of polarized epithelial cells. *Sci. Rep.* **9**, 17079. doi:10.1038/s41598-019-53085-6

Kortebi, M., Milohanic, E., Mitchell, G., Péchoux, C., Prevost, M.-C., Cossart, P. and Bierne, H. (2017). *Listeria monocytogenes* switches from dissemination to persistence by adopting a vacuolar lifestyle in epithelial cells. *PLoS Pathog.* **13**, e1006734. doi:10.1371/journal.ppat.1006734

Kretzschmar, K. and Clevers, H. (2016). Organoids: modeling development and the stem cell niche in a dish. *Dev. Cell* **38**, 590-600. doi:10.1016/j.devcel.2016.08.014

Krokowski, S., Lobato-Márquez, D., Chastanet, A., Pereira, P. M., Angelis, D., Galea, D., Larrouy-Maumus, G., Henriques, R., Spiliotis, E. T., Carballido-López, R. et al. (2018). Septins recognize and entrap dividing bacterial cells for delivery to lysosomes. *Cell Host Microbe* **24**, 866-874.e4. doi:10.1016/j.chom.2018.11.005

Langhorst, M. F., Schaffer, J. and Goetze, B. (2009). Structure brings clarity: Structured illumination microscopy in cell biology. *Biotechnol. J.* **4**, 858-865. doi:10.1002/biot.200900025

Lee, K. M., Choi, K. H. and Ouellette, M. M. (2004). Use of exogenous hTERT to immortalize primary human cells. *Cytotechnology* **45**, 33-38. doi:10.1007/10.1007/s10616-004-5123-3

Leevy, W. M., Serazin, N. and Smith, B. D. (2007). Optical imaging of bacterial infection models. *Drug Discov. Today Dis. Model.* **4**, 91-97. doi:10.1016/j.ddmod.2007.07.001

Lerner, T. R., de Souza Carvalho-Wodarz, C., Repnik, U., Russell, M. R. G., Borel, S., Diedrich, C. R., Rohde, M., Wainwright, H., Collinson, L. M., Wilkinson, R. J. et al. (2016). Lymphatic endothelial cells are a replicative niche for *Mycobacterium tuberculosis*. *J. Clin. Invest.* **126**, 1093-1108. doi:10.1172/JCI83379

Libertucci, J. and Young, V. B. (2019). The role of the microbiota in infectious diseases. *Nat. Microbiol.* **4**, 35-45. doi:10.1038/s41564-018-0278-4

Liehr, T. (2017). In *Fluorescence In Situ Hybridization (FISH) Application Guide* (ed. T. Liehr). Berlin: Springer.

Liu, S., Hatton, M. P., Khandelwal, P. and Sullivan, D. A. (2010). Culture, immortalization, and characterization of human meibomian gland epithelial cells. *Investig. Ophthalmol. Vis. Sci.* **51**, 3993-4005. doi:10.1167/iovs.09-5108

Marrazzo, P., Maccari, S., Taddei, A., Bevan, L., Telford, J., Soriani, M. and Pezzicoli, A. (2016). 3D reconstruction of the human airway mucosa *in vitro* as an experimental model to study NTHi infections. *PLoS ONE* **11**, e0153985. doi:10.1371/journal.pone.0153985

Marrero, B., Messina, J. L. and Heller, R. (2009). Generation of a tumor spheroid in a microgravity environment as a 3D model of melanoma. *Vitr. Cell. Dev. Biol. Anim.* **45**, 523-534. doi:10.1007/s11626-009-9217-2

Maunsbach, A. B. and Afzelius, B. A. (1999). *Biomedical Electron Microscopy*. Elsevier.

McCance, D. J., Kopan, R., Fuchs, E. and Laimins, L. A. (1988). Human papillomavirus type 16 alters human epithelial cell differentiation *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**, 7169-7173. doi:10.1073/pnas.85.19.7169

Medeiros, J. M., Böck, D., Weiss, G. L., Kooger, R., Wepf, R. A. and Pilhofer, M. (2018). Robust workflow and instrumentation for cryo-focused ion beam milling of samples for electron cryotomography. *Ultramicroscopy* **190**, 1-11. doi:10.1016/j.ultramic.2018.04.002

Morise, H., Shimomura, O., Johnson, F. H. and Winant, J. (1974). Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* **13**, 2656-2662. doi:10.1021/bi00709a028

Mostowy, S., Bonazzi, M., Hamon, M. A., Tham, T. N., Mallet, A., Lelek, M., Gouin, E., Demangel, C., Brosch, R., Zimmer, C. et al. (2010). Entrapment of intracytosolic bacteria by septin cage-like structures. *Cell Host Microbe* **8**, 433-444. doi:10.1016/j.chom.2010.10.009

Moter, A. and Göbel, U. B. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Methods* **41**, 85-112. doi:10.1016/S0167-7012(00)00152-4

Nieto, V., Kroken, A. R., Grosser, M. R., Smith, B. E., Metruccio, M. M. E., Hagan, P., Hallsten, M. E., Evans, D. J. and Fleiszig, S. M. J. (2019). Type IV pili can mediate bacterial motility within epithelial cells. *MBio* **10**, e02880-18. doi:10.1128/mBio.02880-18

Noel, G., Baetz, N. W., Staab, J. F., Donowitz, M., Kovbasnjuk, O., Pasetti, M. F. and Zachos, N. C. (2017). A primary human macrophage-enteroid co-culture model to investigate mucosal gut physiology and host-pathogen interactions. *Sci. Rep.* **7**, 46790. doi:10.1038/srep46790

Ono, M., Murakami, T., Kudo, A., Isshiki, M., Sawada, H. and Segawa, A. (2001). Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy. *J. Histochem. Cytochem.* **49**, 305-311. doi:10.1177/002215540104900304

Papazian, D., Würtzen, P. A. and Hansen, S. W. K. (2016). Polarized airway epithelial models for immunological co-culture studies. *Int. Arch. Allergy Immunol.* **170**, 1-21. doi:10.1159/000445833

Paz, A. C., Soleas, J., Poon, J. C. H., Trieu, D., Waddell, T. K. and McGuigan, A. P. (2014). Challenges and opportunities for tissue-engineering polarized epithelium. *Tissue Eng. Part B Rev.* **20**, 56-72. doi:10.1089/ten.teb.2013.0144

Peddie, C. J. and Collinson, L. M. (2014). Exploring the third dimension: volume electron microscopy comes of age. *Micron* **61**, 9-19. doi:10.1016/j.micron.2014.01.009

Pedersen, R. M., Grønnemose, R. B., Stærk, K., Asferg, C. A., Andersen, T. B., Kolmos, H. J., Møller-Jensen, J. and Andersen, T. E. (2018). A method for quantification of epithelium colonization capacity by pathogenic bacteria. *Front. Cell. Infect. Microbiol.* **8**, 16. doi:10.3389/fcimb.2018.00016

Pohl, C., Hermanns, M. I., Uboldi, C., Bock, M., Fuchs, S., Dei-Anang, J., Mayer, E., Kehe, K., Kummer, W. and Kirkpatrick, C. J. (2009). Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. *Eur. J. Pharm. Biopharm.* **72**, 339-349. doi:10.1016/j.ejpb.2008.07.012

Pollack, M., Raubitschek, A. A. and Larrick, J. W. (1987). Human monoclonal antibodies that recognize conserved epitopes in the core-lipid A region of lipopolysaccharides. *J. Clin. Invest.* **79**, 1421-1430. doi:10.1172/JCI112970

Rainey, P. B. and Rainey, K. (2003). Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**, 72-74. doi:10.1038/nature01906

Rajan, S., Cacalano, G., Bryan, R., Ratner, A. J., Sontich, C. U., van Heerckeren, A., Davis, P. and Prince, A. (2000). *Pseudomonas aeruginosa* induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. *Am. J. Respir. Cell Mol. Biol.* **23**, 304-312. doi:10.1165/ajrcmb.23.3.4098

Ram, S., Prabhat, P., Ward, E. S. and Ober, R. J. (2009). Improved single particle localization accuracy with dual objective multifocal plane microscopy. *Opt. Express* **17**, 6881. doi:10.1364/OE.17.006881

Rambhatla, L., Chiu, C.-P., Glickman, R. D. and Rowe-Rendleman, C. (2002). *In vitro* differentiation capacity of telomerase immortalized human RPE cells. *Investig. Ophthalmol. Vis. Sci.* **43**, 1622-1630.

Ramirez, R. D., Sheridan, S., Girard, L., Sato, M., Kim, Y., Pollack, J., Peyton, M., Zou, Y., Kurie, J. M., DiMaio, J. M. et al. (2004). Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res.* **64**, 9027-9034. doi:10.1158/0008-5472.CAN-04-3703

Ravi, M., Paramesh, V., Kaviya, S. R., Anuradha, E. and Paul Solomon, F. D. (2015). 3D cell culture systems: Advantages and applications. *J. Cell. Physiol.* **230**, 16-26. doi:10.1002/jcp.24683

Rayleigh, L. R. S. R. (1896). XV. On the theory of optical images, with special reference to the microscope. *London, Edinburgh, Dublin Philos. Mag. J. Sci.* **42**, 167-195. doi:10.1080/14786449608620902

Ribet, D. and Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect.* **17**, 173-183. doi:10.1016/j.micinf.2015.01.004

Ries, J., Kaplan, C., Platonova, E., Eghlidi, H. and Ewers, H. (2012). A simple, versatile method for GFP-based super-resolution microscopy via nanobodies. *Nat. Methods* **9**, 582-584. doi:10.1038/nmeth.1991

Roberts, T. M., Rudolf, F., Meyer, A., Pellaux, R., Whitehead, E., Panke, S. and Held, M. (2016). Identification and characterisation of a pH-stable GFP. *Sci. Rep.* **6**, 28166. doi:10.1038/srep28166

Russell, M. R. G., Lerner, T. R., Burden, J. J., Nkwe, D. O., Pelchen-Matthews, A., Domart, M.-C., Durgan, J., Weston, A., Jones, M. L., Peddie, C. J. et al. (2017). 3D correlative light and electron microscopy of cultured cells using serial blockface scanning electron microscopy. *J. Cell Sci.* **130**, 278-291. doi:10.1242/jcs.188433

Rybakovsky, E., Valenzano, M. C., Diguilio, K. M., Buleza, N. B., Moskalenko, D. V., Harty, R. N. and Mullin, J. M. (2019). Improving transient transfection efficiency in a differentiated, polar epithelial cell layer. *J. Biomol. Tech.* **30**, 19-24. doi:10.7171/jbt.19-3002-001

Sanger, J. M., Chang, R., Ashton, F., Kaper, J. B. and Sanger, J. W. (1996). Novel form of actin-based motility transports bacteria on the surfaces of infected cells. *Cell Motil. Cytoskelet.* **34**, 279-287. doi:10.1002/(SICI)1097-0169(1996)34:4<279::AID-CM3>3.0.CO;2-3

Sato, T. and Clevers, H. (2015). SnapShot: Growing organoids from stem cells. *Cell* **161**, 1700-1700.e1. doi:10.1016/j.cell.2015.06.028

Schermelleh, L., Ferrand, A., Huser, T., Eggeling, C., Sauer, M., Biehlmaier, O. and Drummen, G. P. C. (2019). Super-resolution microscopy demystified. *Nat. Cell Biol.* **21**, 72-84. doi:10.1038/s41556-018-0251-8

Sebrell, T. A., Sidar, B., Bruns, R., Wilkinson, R. A., Wiedenheft, B., Taylor, P. J., Perrino, B. A., Samuelson, L. C., Wilking, J. N. and Bimczok, D. (2018). Live imaging analysis of human gastric epithelial spheroids reveals spontaneous rupture, rotation and fusion events. *Cell Tissue Res.* **371**, 293-307. doi:10.1007/s00441-017-2726-5

Sebrell, T. A., Hashimi, M., Sidar, B., Wilkinson, R. A., Kirpotina, L., Quinn, M. T., Malkoç, Z., Taylor, P. J., Wilking, J. N. and Bimczok, D. (2019). A novel gastric spheroid co-culture model reveals chemokine-dependent recruitment of human dendritic cells to the gastric epithelium. *CMGH* **8**, 157-171.e3. doi:10.1016/j.jcmgh.2019.02.010

Severi, E., Hood, D. W. and Thomas, G. H. (2007). Sialic acid utilization by bacterial pathogens. *Microbiology* **153**, 2817-2822. doi:10.1099/mic.0.2007/009480-0

Shamir, E. R. and Ewald, A. J. (2014). Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nat. Rev. Mol. Cell Biol.* **15**, 647-664. doi:10.1038/nrm3873

Shinoda, H., Shannon, M. and Nagai, T. (2018). Fluorescent proteins for investigating biological events in acidic environments. *Int. J. Mol. Sci.* **19**, 1548. doi:10.3390/ijms19061548

Sograte-Idrissi, S., Schlichthaerle, T., Duque-Afonso, C. J., Alevra, M., Strauss, S., Moser, T., Jungmann, R., Rizzoli, S. O. and Opazo, F. (2020). Circumvention of common labelling artefacts using secondary nanobodies. *Nanoscale* **12**, 10226-10239. doi:10.1039/D0NR00227E

Sonoda, S., Spee, C., Barron, E., Ryan, S. J., Kannan, R. and Hinton, D. R. (2009). A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. *Nat. Protoc.* **4**, 662-673. doi:10.1038/nprot.2009.33

Spector, D. L., Goldman, R. D. and Leinwand, L. A. (1998). Fluorescence *in situ* hybridization to DNA. In *Cells: a laboratory manual*. Cold Spring Harbor Laboratory Press.

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L. and Hickman, J. J. (2015). TEER measurement techniques for *in vitro* barrier model systems. *J. Lab. Autom.* **20**, 107-126. doi:10.1177/2211068214561025

Starner, T. D., Zhang, N., Kim, G., Apicella, M. A. and McCray, P. B. Jr. (2006). *Haemophilus influenzae* forms biofilms on airway epithelia: implications in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **174**, 213-220. doi:10.1164/rccm.200509-1459OC

Steinke, M., Gross, R., Walles, H., Gangnus, R., Schütze, K. and Walles, T. (2014). An engineered 3D human airway mucosa model based on an SIS scaffold. *Biomaterials* **35**, 7355-7362. doi:10.1016/j.biomaterials.2014.05.031

Stoddart, M. J. (2011). Cell viability assays: introduction. *Methods Mol. Biol.* **740**, 1-6. doi:10.1007/978-1-61779-108-6_1

Sullivan, M. J. and Ulett, G. C. (2018). Stable expression of modified green fluorescent protein in group B streptococci to enable visualization in experimental systems. *Appl. Environ. Microbiol.* **84**, e01262-18. doi:10.1128/AEM.01262-18

Uotani, T., Murakami, K., Uchida, T., Tanaka, S., Nagashima, H., Zeng, X. L., Akada, J., Estes, M. K., Graham, D. Y. and Yamaoka, Y. (2019). Changes of tight junction and interleukin-8 expression using a human gastroid monolayer model of *Helicobacter pylori* infection. *Helicobacter* **24**, e12583. doi:10.1111/hel.12583

Van Engelenburg, S. B. and Palmer, A. E. (2010). Imaging type-III secretion reveals dynamics and spatial segregation of *Salmonella* effectors. *Nat. Methods* **7**, 325-330. doi:10.1038/nmeth.1437

Van Wijk, S. J. L., Fricke, F., Herhaus, L., Gupta, J., Hötte, K., Pampaloni, F., Grumati, P., Kaulich, M., Sou, Y.-S., Komatsu, M. et al. (2017). Linear ubiquitination of cytosolic *Salmonella Typhimurium* activates NF-κB and restricts bacterial proliferation. *Nat. Microbiol.* **2**, 17066. doi:10.1038/nmicrobiol.2017.66

Vangindertael, J., Camacho, R., Sempels, W., Mizuno, H., Dedecker, P. and Janssen, K. P. F. (2018). An introduction to optical super-resolution microscopy for the adventurous biologist. *Methods Appl. Fluoresc.* **6**, 022003. doi:10.1088/2050-6120/aaae0c

Vayssier-Taussat, M., Albina, E., Citti, C., Cosson, J.-F., Jacques, M.-A., Lebrun, M.-H., Le Loir, Y., Ogliastro, M., Petit, M.-A., Roumagnac, P. et al. (2014). Shifting the paradigm from pathogens to pathobiome new concepts in the light of meta-omics. *Front. Cell. Infect. Microbiol.* **5**, 1-7. doi:10.3389/fcimb.2014.00029

Velle, K. B. and Campellone, K. G. (2017). Extracellular motility and cell-to-cell transmission of enterohemorrhagic *E. coli* is driven by EspFU-mediated actin assembly. *PLoS Pathog.* **13**, e1006501. doi:10.1371/journal.ppat.1006501

Walker, W. T., Jackson, C. L., Allan, R. N., Collins, S. A., Kelso, M. J., Rineh, A., Yepuri, N. R., Nicholas, B., Lau, L., Johnston, D. et al. (2017). Primary ciliary dyskinesia ciliated airway cells show increased susceptibility to *Haemophilus influenzae* biofilm formation. *Eur. Respir. J.* **50**, 1700612. doi:10.1183/13993003.00612-2017

Ward, W. W., Prentice, H. J., Roth, A. F., Cody, C. W. and Reeves, S. C. (1982). Spectral perturbations of the *Aequorea* green–fluorescent protein. *Photochem. Photobiol.* **35**, 803-808. doi:10.1111/j.1751-1097.1982.tb02651.x

Weight, C. M., Venturini, C., Pojar, S., Jochems, S. P., Reiné, J., Nikolaou, E., Solórzano, C., Noursadeghi, M., Brown, J. S., Ferreira, D. M. et al. (2019). Microinvasion by *Streptococcus pneumoniae* induces epithelial innate immunity during colonisation at the human mucosal surface. *Nat. Commun.* **10**, 3060. doi:10.1038/s41467-019-11005-2

Winey, M., Meehl, J. B., O'Toole, E. T. and Giddings, T. H. (2014). Conventional transmission electron microscopy. *Mol. Biol. Cell* **25**, 319-323. doi:10.1091/mbc.e12-12-0863

Yonker, L. M., Mou, H., Chu, K. K., Pazos, M. A., Leung, H., Cui, D., Ryu, J., Hibbler, R. M., Eaton, A. D., Ford, T. N. et al. (2017). Development of a primary human co-culture model of inflamed airway mucosa. *Sci. Rep.* **7**, 8182. doi:10.1038/s41598-017-08567-w

Young, M. R. (1961). Principles and technique of fluorescence microscopy. *J. Cell Sci.*, **s3-s102**, 419-449.

Young, A. M., Minson, M., McQuate, S. E. and Palmer, A. E. (2017). Optimized fluorescence complementation platform for visualizing *Salmonella* effector proteins reveals distinctly different intracellular niches in different cell types. *ACS Infect. Dis.* **3**, 575-584. doi:10.1021/acsinfecdis.7b00052