Single-cell Imaging Assay for Rapid Antimicrobial Resistance Testing

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Abstract

Antimicrobial resistance (AMR) is arguably one of the biggest healthcare challenges in the new century, the constant rise of which presents extra threat, projecting to cause 10 million deaths and billions of dollars in cost by 2050 if no action is taken. Drug-resistant pathogens have no border and can travel to any corner of the world, regardless of wealth or distance. One of the biggest challenges in addressing this rise of AMR is the low speed of current diagnostic methods, forcing physicians to prescribe inappropriate or unnecessary antibiotics before a diagnosis is reached, and such misuse inevitably exacerbates the rise of AMR. In this project, a proof-of-principle imaging assay was developed to significantly accelerate the procedure to reach a correct diagnosis. This assay used fluorescence *in situ* hybridisation (FISH) to detect RNA targets that led to the identification of bacterial speciation and the bacteria's resistance to certain antibiotics. It was validated with laboratory and clinical isolates of *E. coli* with promising results. This project paved the way to a multiplex assay that would generate robust and reliable results within one hour.

CONTENTS

LIST OF ABBREVIATIONS

ADE	Adverse drug event
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility test
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
COVID-19	Coronavirus disease of 2019
CSF	Cerebrospinal fluid
EEA	European Economic Area
ECDC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum β-lactamase
EU	European Union
FISH	Fluorescence *in situ* hybridisation
FOV	Field of view
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
PBP	Penicillin-binding protein
PLL	Poly-L-lysine
PNA	Peptide nucleic acid

1 BACKGROUND

1.1 Antimicrobial Resistance (AMR)

1.1.1 Rising of antimicrobial resistance

Since the discovery of the first antibiotic, penicillin, in 1929, development of antimicrobials has transformed modern medicine, reducing morbidity rate and increasing life span, and saving millions of lives. However, almost as soon as antimicrobials were used in clinics, emergence of drug resistance has been observed. An arms race between development of drugs and bacteria's development of resistance to these drugs has been ever so fierce.

Almost a century later, infections with resistant pathogens account for 25,000 deaths in the European Union, 35,000 deaths in the United States, and over 700,000 deaths worldwide every year (1, 2).

The so-called superbugs, which are resistant to multiple drugs, are already among us. If nothing is done to prevent further emergence and spread, antimicrobial resistance is projected to account for 10 million death tolls each year worldwide by 2050, costing more than $100 trillion in the next 30 years (3), a number that urges immediate and collaborative interventions.

Compared to the western world, it is even more devastating in less developed countries due to a lack of regulation and preventative measures. Their already alarmingly

high rates of resistant infections (e.g., a study showed 40-60% in Brazil, Indonesia, and Russia) together with a 4-7 times faster projected growing rate (4), presents tremendous worries to the world. Much like the viruses responsible for coronavirus disease of 2019 (COVID-19), infectious bacteria and resistant pathogens are not restricted by geological or social-economic boundaries.

The rise of AMR is largely fuelled by the overuse and misuse of antimicrobials: 30-50% antibiotic prescription in clinics are suboptimal, an estimate given by both the European Centre for Disease Prevention and Control (ECDC) and Centers for Disease Control and Prevention (CDC) (1, 2). In addition to therapeutic use for humans, antimicrobials are also used for animal and fish farming and also as pesticides for crops (5, 6). With far more relaxed regulation concerning animal and agricultural use, large quantities of antimicrobials are released into the environment, exacerbating the development of resistance, which inevitably returns to our communities.

Being resistant to even one antibiotic can make bacteria dangerous. When first-line antibiotics (usually broad-spectrum antibiotics like amoxicillin) fail, second-line antibiotics may cause adverse drug events (ADE) including toxicity and allergic reactions, with the young and old being the most affected. An ECDC study in 2015 found 46% of such ADE involved in children who were sent to emergency rooms (7). If last-line antibiotics fail to fight the infection, it would mean no viable treatment thus the end for those infected. Without effective antibiotics, small cuts and minor injuries can pose life-threatening danger to humans, let alone management

of the more advanced medical conditions like surgeries, treatment of cancer, and many other chronic diseases.

To make matters worse, the speed of developing new and effective drugs is much slower than the emergence rate of antibiotic resistance bacteria strains. From 1990 to 2017, 78% drug companies have reduced funding for antibiotic development (8). In 2010, only four major pharmaceutical companies have a division for antibiotic development (9). In addition to the difficulty and immense timescale in research and development, the reduction in investment can also be attributed to the marginal profit of antibiotics compared to drugs that treat cancers or chronic heart diseases. Even if a new drug is developed, there is no guarantee of effectiveness towards all common and hard-to-fight bacteria, and more sadly, the countdown for its effectiveness has begun.

1.1.2 Development of antibiotics

To address the challenge of rising AMR, we first need to understand how antibiotics work. Since the discovery of penicillin in 1929, antibiotics have been discovered and synthesised to fight bacterial infections by killing or inhibiting growth of bacteria. There are four main modes of action for inhibiting bacteria growth (**Figure 1**):

1) *Inhibition of cell wall synthesis*

β-lactams and glycopeptides are the two main classes of antibiotics that disrupt the biosynthesis of bacterial cell walls. β-lactams, such as penicillin, bind to the

enzymes (PBPs, penicillin-binding proteins) involved in the last stage of peptidoglycan formation. As peptidoglycan is an essential component of cell walls and it cannot be assembled properly, cell walls are weakened as a result which then leads to cell lysis (10). Glycopeptides, such as vancomycin, are bulky molecules that bind and occupy the substrates that are important for cell wall synthesis and thus prevent formation of cell walls.

2) Inhibition of protein synthesis

The major groups of antibiotics that inhibit protein synthesis include aminoglycosides, chloramphenicol, and tetracyclines, all of which are 'broad-spectrum' for their activity against a variety of bacteria. By binding to different sites on the ribosomes, aminoglycosides disrupt the initiation and elongation steps in protein synthesis leading to formation of incorrect amino acids and eventually cell deaths (11), while tetracyclines and chloramphenicol disrupt peptide chain elongation step in protein synthesis (12, 13). Tetracyclines are the second most consumed antibiotic globally after penicillin because of its relative safety and low cost (14), whereas chloramphenicol is reserved for only critical conditions due to its serious side-effects and increased resistance.

3) Inhibition of nucleic acid synthesis

Quinolones is the representative class of antibiotics that inhibits synthesis of bacteria's genetic material. Quinolones inhibit two enzymes (DNA gyrase and

topoisomerase IV) essential in DNA replication from action (15, 16). Unfortunately, resistance to quinolones is rapidly involving.

4) Modification of microbes' energy metabolism

Some antibiotics can also modify metabolism pathways of bacteria. As an example, antibiotic sulphonamides don't kill bacteria but prevent growth by disrupting the biosynthesis of folic acid, which is required for bacterial cell growth (17).

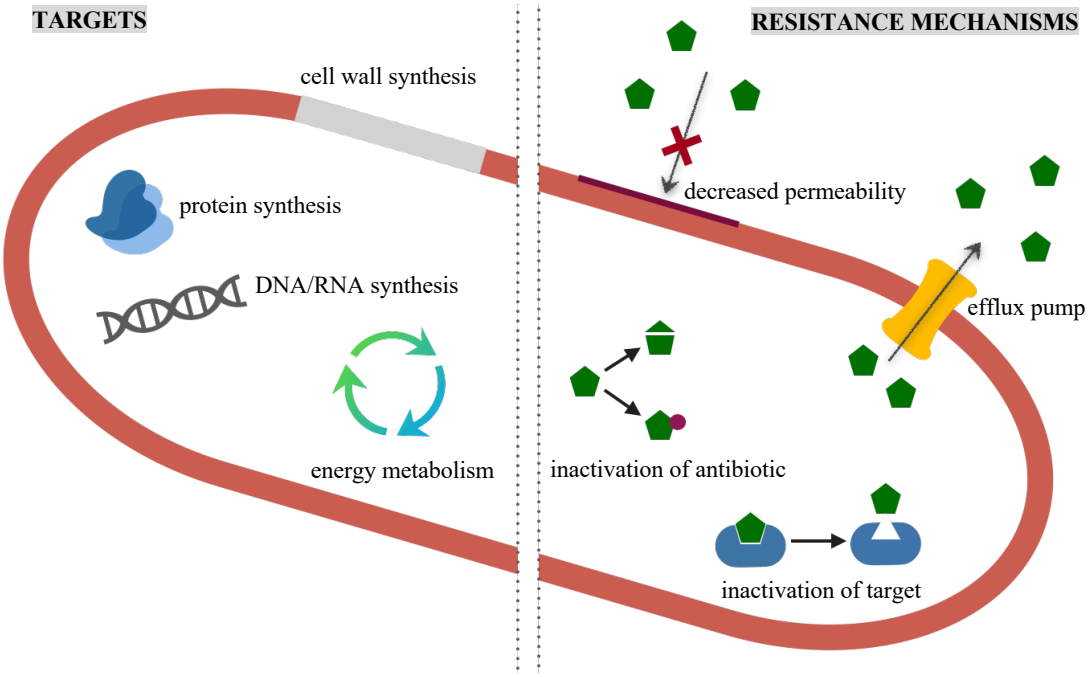

Figure 1 ***Targets of antibiotics (left) and resistance mechanisms of bacteria (right).*** *Antibiotics can inhibit cell growth by inhibiting cell wall synthesis, DNA/RNA synthesis, protein synthesis, or by modifying energy metabolisms such as the metabolism of folic acid. Bacteria can develop drugs resistance mechanisms including decreased cell permeability, inactivation of drugs, inactivation of drug targets, and efflux pumps.*

1.1.3 Development of resistance mechanisms

Almost as soon as there were new antibiotics, resistant strains of bacteria have emerged. Resistance to penicillin was recorded even before it was used therapeutically (18).

Some bacteria can be intrinsically resistant as a mechanism to protect themselves from the antibiotics they produce (19). Otherwise, resistance results from gene mutations or acquisition of these mutated genes. There are three ways of acquiring resistance genes: by inheritance from parent cells, by *de novo* mutagenesis, or by horizontal transmission from other bacteria or even the environment via mobile DNA elements such as plasmids. It means that bacteria don't have to be exposed to antibiotics to develop resistance.

Resistance to antibiotics can be conferred by one or a combination of the following main mechanisms (**Figure 1**):

1) Inactivation of antibiotics

The most common way of inactivating antibiotics is by expressing enzymes that alter the drug compounds, such as β-lactamase that breaks down β-lactam antibiotics. Extended-spectrum β-lactamases (ESBL) - producing *Escherichia coli (E. coli)* have become a major problem as they are resistant to a range of antibiotics. As a result, empirical treatment becomes ineffective, in some cases mortality rate of typical *E. coli* bacteraemia doubles (20), and such resistance is still increasing at an alarming rate (21).

2) Modification of the antibiotic's targets

Another way to make the drugs less effective is to alter its binding site in the cells, such as the PBPs. Susceptible *Staphylococcus aureus* (*S. aureus*) strains have four normal PBPs; however, in resistant *S. aureus* strains PBP2a, a unique protein that has low affinity for β-lactams, is induced and can therefore continue cell wall synthesis without being disrupted. PBP2a is encoded by *mec*A gene which is believed to have given rise to the emergence of methicillin-resistance *S. aureus* (MRSA). MRSA has resistance to a multitude of antibiotics and is one of the most difficult-to-treat infections. Initially emerged from hospitals, dangerous strains like MRSA are now prevalent even in domestic homes (22).

3) Decreased cell permeability

Decreased permeability in cell walls and membranes would restrict the entrance of antibiotics into the cell body. One of the mechanisms of resistance to aminoglycoside is an example of this: changes in the outer membrane proteins, through which antibiotic molecules diffuse inside the cell, can lead to decreased susceptibility to many antibiotics (23).

4) Active efflux pumps

Once antibiotics have entered the cells, efflux pumps could actively remove them from the cells. Efflux is an intrinsic mechanism in both susceptible and resistant bacteria; mutations can increase the number or efficiency of these efflux pump proteins (23, 24).

1.2 Existing Methods

Diagnostic tests are as important as antimicrobials themselves, as they identify the causative pathogens and their susceptibility to antimicrobials. The faster the test, the faster physicians can prescribe the optimal treatments, thus improving patient outcomes.

1.2.1 Bacterial speciation

For the first part of a diagnosis, the causative bacteria species need to be identified. Traditionally, bacteria are identified by a combination of the following methods: examination of colony morphology, microscopy (cellular morphology), and a series of biochemical assays. However, these tests depend on culturing in advance which is a very time-consuming process and remains a big hurdle in fast identification.

Molecular diagnostic methods such as polymerase chain reaction (PCR), whole genome sequencing, and hybridisation of nucleic acids are also used nowadays providing much higher specificity than conventional biochemical assays. Whole-genome sequencing has been applied to study several MRSA strains. However, the methodology is expensive and depends heavily on robust software for analysis.

Since 2010, development of MALDI–TOF MS (matrix assisted laser desorption/ionization-time of flight mass spectrometry mass spectrometry) has revolutionised how clinical laboratories identify bacteria. MALDI-TOF MS is mostly used

together with biochemical tests for result confirmation, as some species are difficult to be differentiated by it (25).

1.2.2 Antimicrobial susceptibility tests (AST)

For the second part of diagnosis, the causative bacteria are tested against an array of antibiotics to determine which would be effective at preventing bacteria growth. Antimicrobial susceptibility tests (AST), the clinical standard diagnostic tools, are *in vitro* methods that test for the response of clinical isolates to antibiotic treatments. There are two types of AST – phenotypic and genotypic – depending on what parameters are taken as metric for diagnosis.

Phenotypic ASTs directly assess whether antibiotics inhibit bacteria growth, with metrics being morphological changes of bacterial cells or ability to proliferate as a colony. Traditional methods include disk diffusion (26) and broth microdilution (27). These standardised tests have been used in clinics for many decades and are able to generate robust and relatively quantitative results: after incubating bacteria isolates with a range of antibiotics of a series of concentrations, appearance of growth inhibition zones on solid agar plates, or a change in optical density (OD) in liquid cultures, are usually indications of susceptibility towards a certain antibiotic (28). Then the tested bacterium is classified as 'susceptible', 'intermediate', or 'resistant' according to criteria set out by the Clinical and Laboratory standards Institute (CLSI) or relevant authorities (29). In addition, a minimum inhibitory

concentration (MIC) – i.e., the lowest concentration of antibiotic needed to inhibit bacterial growth – is determined for each antibiotic on each of the tested strains.

A major limitation for these traditional ASTs is the slow speed: it takes at least 24-48 hours to obtain clinical isolates from clinical specimens, followed by 16-24 hours of manual biochemical tests (**Figure 2**). For traditional biochemical tests, the lack of sensitivity constitutes the bottleneck. For broth microdilution, for example, to detect any change in the liquid culture's OD, the concentration of bacteria needs to reach 10^7 CFU/ml (colony forming unit /ml), which can take up to two days.

It has been shown that optimisation of antimicrobial therapy is of crucial importance in the first 6-12 hours of infection, particularly for life-threatening infections, such as when urinary tract infection develops into sepsis. Each hour of delay in the administration of antimicrobials is found to decrease the septic patient's survival rate by an average of 7.6% (30). Although generating robust results, the slow speed and labour-intensity of these tests restrict them to central laboratory testing rather than near-patient settings.

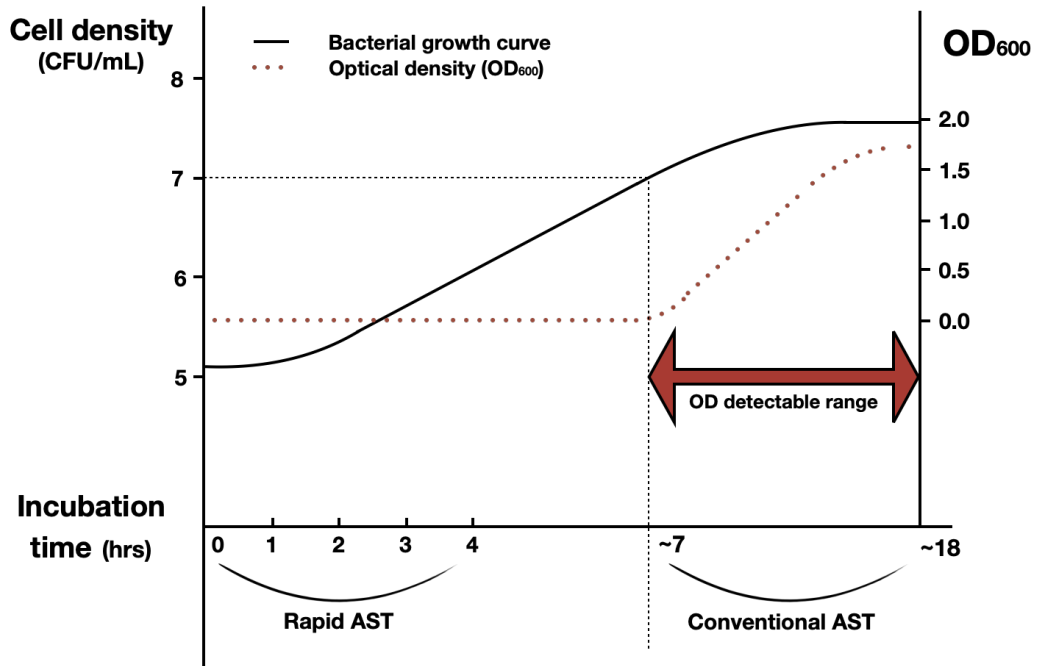

Figure 2 Bacteria growth curve in relation to changes in optical density (OD) of liquid cultures for clinical isolates. *In a typical clinical setting, a blood sample taken from a patient with symptoms of blood-borne infections possess no more than 10^3 CFU/ml bacterial cells, among billions of human blood cells and other debris. It takes 48 hours to process blood samples, obtain and identify bacteria isolates, and grow bacteria to a concentration of 10^5 CFU/ml. In order to detect a change in the OD of the liquid culture, concentration of bacteria has to reach 10^7 CFU/ml, requiring a further 6-8 hours of incubation. Only at this stage can conventional ASTs be performed which take another 8-24 hours. Adapted from "A rapid antimicrobial susceptibility test based on single-cell morphological analysis" by J. Choi, J. Yoo, M. Lee, … S. Kwon, 2014, Science Translational Medicine,* ***16****, 267-274. Copyright 2014 by the American Association for the Advancement of Science.*

Several rapid ASTs have been recently developed. Many accelerate the process by utilising microfluidics to test small populations of bacteria in confined volumes (31-36). Previous studies used cell death (31), cell density (32), metabolic activity (33), or a panel of morphological changes (34, 35) as indications of antibiotic susceptibility. One particularly fast AST measured changes in cell length and could produce an accurate diagnosis within 30 minutes (36).

However, these phenotypic ASTs rely on the assumption that the bacteria strains are isolated and their identity are known, which takes at least 24 hours to achieve.

Most clinical microbiologists are unwilling to base their conclusion solely on such AST tests without bacterial speciation (37). Moreover, they only tested a limited range of parameters, cell strains, and antibiotics. Bacteria can have very distinct responses to different antibiotics so a comprehensive test would involve multiplexed setup and analysis (38, 39).

Genotypic ASTs, based on amplification (PCR), hybridisation, or sequencing, detect the existence of genetic markers (genes, plasmids, etc.) that are known to be associated with AMR. Genotypic tests can be much faster and have shown great potential in detecting resistance in certain Gram-positive bacteria.

There are several limitations with genotypic ASTs. First of all, presence of AMR genes doesn't necessarily correlate with its antimicrobial susceptibility. Some genes may not be expressed enough to cause resistance, whereas some may not be expressed at all. AMR genes are not restricted to reside on the genome; mobile genetic elements - such as plasmids, transposons, and integrons - are also responsible for carrying AMR genes and are capable of horizontally transferring them to other bacteria populations or even genera. Secondly, absence of an AMR gene doesn't guarantee its susceptibility to the antibiotics. One bacterium can possess multiple mechanisms for resistance, and multiple genetic mutations can alter the same enzyme that contributes to the same resistance mechanism. There are also persister bacteria that survive antibiotics by stopping growth transiently (40). In addition, not only are genotypic methods relatively more expensive, but they have also limited

capability of detecting only known resistance genes. New resistant genes emerge quickly as the use of antibiotics pushes bacteria to constantly mutate.

In summary, phenotypic and genotypic ASTs all have advantages and limitations, and so both types of tests would be needed to reach a mutually conclusive diagnosis.

1.2.3 Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation (FISH) is a simple but powerful tool because it can be used to satisfy multiple needs of bacterial speciation and AMR detection.

In situ hybridisation, a staining procedure first developed in 1969, is based on the complementary binding of a labelled nucleotide probe to a target nucleic acid sequence inside the organism (41, 42). Initially radioactively labelled probes were used, but as they were hazardous, costly, and required long exposure times, fluorescently labelled probes were introduced for imaging 11 years later.

A standard FISH protocol includes the following steps: fixation and permeabilisation of target cells, hybridisation of fluorophore-labelled probes to the target sequences, removal of excess probes, and lastly imaging using fluorescence microscopy.

Designing FISH probes is of critical importance. Probes are usually short (16-25 oligonucleotides) because as probe length decreases diffusion coefficient increases (43, 44). Shorter probe size was also found to increase signal-to-noise ratio (45). More recently peptide nucleic acid (PNA), analogous to DNA, has been developed

for FISH that can hybridise with higher specificity. However, PNA is many times more expensive than DNA for making probes because it is patented. Sequence for the probes can be obtained in several well-established databases for RNA targets such as SILVA rRNA database (46) and Ribosomal Database Project (47).

FISH enables direct examination of genetic materials in their natural environment, as opposed to routine biochemical assays that rely on target extraction. On the one hand, FISH accelerates the process and reduces time by skipping the extraction step. On the other hand, FISH preserves the cell integrity and also reveals targets' location, distribution, association with other compounds, and morphology of the studied organisms.

There are many advantages of FISH. Because FISH doesn't require extraction of the intracellular targets, examination of the targets in their natural environment provide additional information on the cell morphology and molecule spatial distribution. FISH is able to identify bacteria on the level from domains to species, and it can also be used to detect bacteria that are difficult to culture or difficult to identify by other tests, such as facultative or obligate bacteria. When integrated with microfluidics, FISH has the potential to be automated and thus significantly reduce labour and time required.

One of the biggest limitations of FISH is that interpreting its results requires experience, as there is no standard protocol for analysis. Other limitations include the relatively high costs for labelled probes and advanced single-molecule fluorescence

microscopes, the time needed to prepare samples and image analysis, as well as the need to optimise for different bacteria species and sample types. It is more difficult to apply FISH to mRNAs since they have a much lower copy number than rRNA and would require more advanced microscopy equipment and analyses.

Some recent advances in FISH that are relevant to this thesis include single-molecule FISH (smFISH), combinatorial labelling and spectral imaging (CLASI)-FISH (48), multiplexed error-robust FISH (MERFISH) (49).

Single-molecule FISH allows detection of low copies of single mRNA and was first developed in 1998 (50). In smFISH, one mRNA is targeted by multiple short probes at various locations, and each probe is labelled with a different fluorophore. Co-localisation of fluorescence signals from multiple fluorophores would indicate successful detection of the target mRNA, whereas non-specific binding of probes would fail to co-localise and thus their false-positive signals would be disregarded, effectively improving specificity of FISH.

CLASI-FISH, a FISH method that recorded images spectrally and used linear unmixing algorithms, was able to differentiate over a dozen of laboratory-grown human oral microbes in an artificial mixture (48).

MERFISH, a highly multiplexed single-molecule FISH method able to detect 100+ RNAs in single mammalian cells (49). MERFISH also used combinatorial labelling with sequential hybridisation – 16 rounds for 140 RNA targets with error correction and 14 rounds for 1001 RNA targets without correction. However, one challenge

with MERFISH was that as the number of hybridisation rounds increased, both the number of detectable targets and the detection errors rose exponentially.

2 INTRODUCTION

2.1 Project Summary

2.1.1 Project aims

To combat the fast rise in AMR and the detrimental consequences it presents, collaboration across all nations and sectors are required to preserve the efficacy of existing drugs while developing new ones. This project addressed the urgent need to slow down the AMR rise so existing antibiotics remain effective as well as giving time for new drugs to be developed and tested. Because such AMR rise is aggravated by the over-prescription and mis-prescription of antibiotics before a correct diagnosis is reached (normally in two days), an imaging assay was proposed that would significantly accelerate the diagnostic process and generate results within one hour.

For this purpose, the developed imaging assay must satisfy two requirements: 1) be able to identify the species of the causative bacteria, and 2) be able to determine which antibiotics would be effective or ineffective at inhibiting growth of the causative bacteria. The long-term aim would be to enable identification of 10 major pathogens based on 16S rRNA and 20 major AMR mRNAs.

The ultimate goal for this project was to obtain the proof of principle for a rapid imaging assay at the single-cell level, allowing for RNA-based bacterial identification and a rapid genotypic AST. More specifically, fluorescence *in situ* hybridization was used, targeting at a region on the 16S rRNA for bacterial identification and one messenger RNA (mRNA) for antimicrobial susceptibility profile of the bacteria. In addition, the spatial distribution of molecules in target was also visualised and confirmed with literature. The assay was conducted on the same

population of bacteria on the same instrument using fluorescence microscopy, followed by semi-automated imaging analysis. The assay was validated with pathogenic clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*, both belonging to the Gram-negative Gammaproteobacteria group. This paved the way for a multiplex imaging assay combining both genotypic and phenotypic ASTs that our research group is able to take forward.

2.1.2 Collaboration

Clinical collaborators at the John Radcliffe Hospital, Professor Derrick Crook, Dr Monique Andersson, and Dr Nicole Stoesser provided clinical isolates of bacteria, as well as conducting the standard clinical tests for reference. Dr Stoesser and her team supervised my work in the clinical laboratory in addition to pre-processing the samples. Furthermore, their expertise in bacterial pathogens and diagnostics was valuable throughout the duration of this project.

2.1.3 Significance

Success of such rapid diagnostic assay is of great clinical significance. First of all, it can narrow and optimise antimicrobial therapy for patients. Using a treatment with proven antibiotic activity against the targeted pathogen, rather than using broad-spectrum antibiotics, not only improves patient outcomes but also reduces the risk of adverse drug events. Secondly, it can quickly identify infected patients with resistant pathogens who don't show symptoms, and an immediate isolation of which can prevent outbreaks within the healthcare facility. Thirdly, a rapid diagnostic test can facilitate antimicrobial stewardship both regionally and more broadly, as well as epidemiological surveillance, which further contributes to the management of rising antimicrobial resistance.

Development of such rapid diagnostic assay is also beneficial to pharmaceutical development. If incorporated in clinical trials, this can accelerate the rate of which new drugs can be validated

and introduced in therapeutic treatment. Maintaining the efficacy of existing antibiotics also avoids wasting the immense amount of time and resources invested in developing these drugs. Compared to broad-spectrum antibiotics, narrow-spectrum antibiotics for a specific pathogen or pathogen group are easier to discover and often cheaper and faster to develop.

2.2 Experimental Design

2.2.1 Fluorescence *in situ* hybridisation

- *RNA Targets*

The first target was a region on 16S ribosomal RNA (rRNA) that is conserved in the domain of *Bacteria* (51). 16S rRNA was a good target as there can be, in rich media, up to 55,000 ribosomes in a single cell right before division (52). It also served as a positive control for subsequent experiments with new targets or probes.

Ribosomes are micro-machines in cells that specialise in messenger RNA (mRNA) translation and protein synthesis. All prokaryotes have 70S (S = Svedberg units, a measure of particle size) ribosomes consisting of two subunits – large (50S) subunit and small (30S) subunit – which only bind to each other in the event of translation (**Figure 3**). The 30S subunit is composed of 16S rRNA and proteins. 16S rRNA contains the sequence that is complementary to the ribosome binding site on mRNA, making it essential for the initiation of translation.

Within the approximately 1500 base pairs of bacterial 16S rRNA gene, there are nine hypervariable regions (each with 30 to 100 base pairs), between which are sequences that have been conserved throughout evolution (53). The 16S rRNA sequences have been extensively used for phylogenetic studies. In particular, DNA probe EUB338 was first designed in 1990 (54), and henceforth extensively studied, to target a portion of the 16S rRNA (**Figure 3**) that is conserved in the domain of *Bacteria* (51).

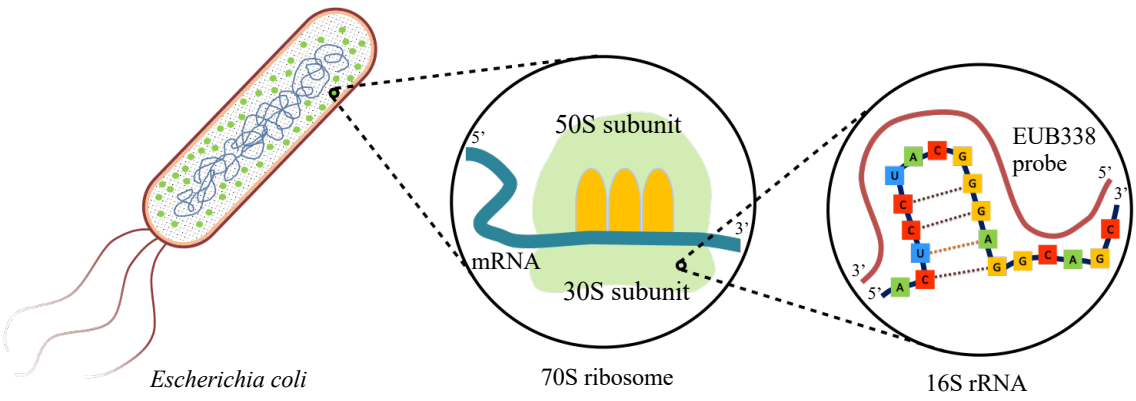

Figure 3 Illustration of a ribosome in E. coli and one region on the 16S rRNA that were studied and used as a target. *(Left) One E. coli cell as a rod-shaped cell with flagella on the outside and thousands of ribosomes on the inside. (Middle) A 70S ribosome consists of two subunits, 50S and 30S subunits, which bind together during translation. (Right) Binding of EUB338 probe to the target sequence (location 338-355 on 16S rRNA).*

Analysis of the hypervariable regions has been one of the most common tools for identification and characterization of larger taxonomic levels in the bacterial community, such as domain, phyla, classes, and orders. The 16S rRNA gene sequences for most bacteria have been documented and are available in several public databases such as EzTaxon-e (55), SILVA (46), and Ribosomal Database Project (47). These databases provide a foundation for targeting species-specific sequences for bacterial speciation.

As for AMR-associated mRNAs, β-lactamase mRNA was chosen as the first target because it is one of the most common resistance mechanisms found in bacteria. β-lactamase is an enzyme that hydrolyses the amide bond in β-lactam rings in antibiotics like penicillins, and thus providing bacteria resistance against these antibiotics.

This project aimed to develop a proof-of-principle imaging assay and so only two targets were used and tested. For future work, more hypervariable regions on 16S rRNA and a number of other AMR mRNAs would be included in the target library to achieve the ultimate goal of identifying 10 major pathogens based on 16S rRNA and 20 major AMR mRNAs.

- *Fluorescence labelling*

Biological molecules like RNA do not emit enough fluorescence to be detected, so in order to be visualised, these biomolecules need to be labelled with a fluorescence molecule, such as a fluorescence dye or fluorescence protein. Fluorescence molecules absorb light of specific wavelengths and emit light of specific wavelengths. Because there is a discrepancy between the absorption and emission wavelengths, called Stokes Shift, optical filters can be used to separate the emission lights.

Organic fluorophores were used for the RNA FISH assay because of their small size, brightness and photostability. Cyanine 3 (Cy3) was chosen as the first and main fluorophore in the experiments for its brightness and relative stability of biophysical properties; cyanine 5 (Cy5) was chosen to be the second fluorophore for multi-colour detection (**Figure 4**).

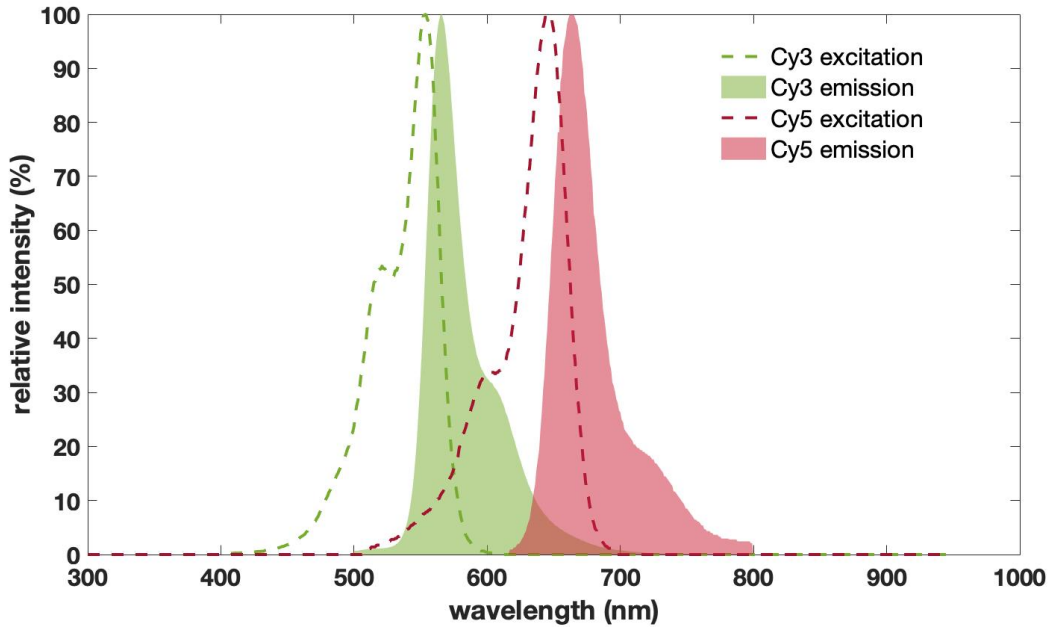

Figure 4 Absorption and emission spectra of Cy3 and Cy5. *Fluorescence molecules absorb light of specific wavelengths and emit light that have shifted towards higher wavelengths.*

- *Probe design*

The probes designed were categorised into primary and secondary probes (**Figure 5**). Primary probes were sequences that directly bound to the targets, whereas secondary probes were sequences that bound directly to primary probes. Some primary probes consisted of a central recognition part that directly bound to the target sequences, and two flanking sequences which were designed so that they wouldn't bind to anywhere in the cell (56). Secondary probes would bind to one flanking sequence on either side of the primary probes. Probes could be labelled with a fluorophore or without.

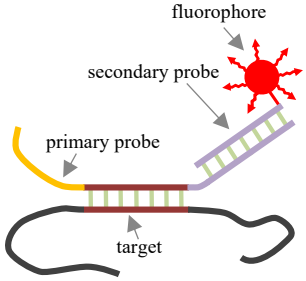

Figure 5 Schematics of primary and secondary probes.

All probes were custom made by Sigma-Aldrich, at a 200nmole synthesis scale for each probe. Probe sequences used in the experiments were listed in **Table 1**. For the target 16S rRNA, primary probes' central recognition part utilised sequence of DNA probe EUB338 (54). For the target β-lactamase mRNA, primary probe's central recognition part utilised a sequence from literature (57).

Table 1 DNA probes designed and used in the experiments. Subscripts of probe names denoted lengths of the probes. In square brackets of probe sequences were the labelled fluorophores.

	Probe name	Probe sequence
Primary	EUB_{18}-Cy3	5'-[Cy3] GCTGCCTCCCGTAGGAGT-3'
	EUB_{58}-Cy5	5'-TACGGCCCCAAGGTCCAAACGCTGCCTCCCGTAGGAG-TGATGGCGCCTCATCCCTGAA [Cy5]-3'
	EUB_{58}	5'-TACGGCCCCAAGGTCCAAACGCTGCCTCCCGTAGGAG-TGATGGCGCCTCATCCCTGAA-3'
	$nonEUB_{58}$	5'-TACGGCCCCAAGGTCCAAACACTCCTACGG-GAGGCAGCGATGGCGCCTCATCCCTGAA-3'
	Amp_{63}	5'-TACGGCCCCAAGGTCCAAACCAGCATCTTTTACTTTCAC-CAGCGATGGCGCCTCATCCCTGAA-3'
Secondary	R1-Cy3	5'-[Cy3] TTCAGGGATGAGGCGCCATC-3'
	R2-Cy3	5'-GTTTGGACCTTGGGGCCGTA [Cy3]-3'
	R1-Cy5	5'-[Cy5] TTCAGGGATGAGGCGCCATC-3'

- *Controls*

To make sure that hybridisation worked, several control experiments needed to be established in the beginning: one on primary hybridisation, one on secondary hybridisation, and lastly on sequential hybridisation of the same field of view.

2.2.3 Multicolour coding system

The FISH assay adapted MERFISH, a highly multiplexed single-molecule FISH method able to detect >100 mRNAs in mammalian cells (49), to rRNA/mRNA detection in bacteria cells. **Figure 6** shows that, even with one fluorophore, there can be as many targets as there are coding sequences, making it much more feasible and cost-effective than using multiple fluorophores. With two or more fluorophores, a multicolour coding system can be implemented for more targets while minimising errors.

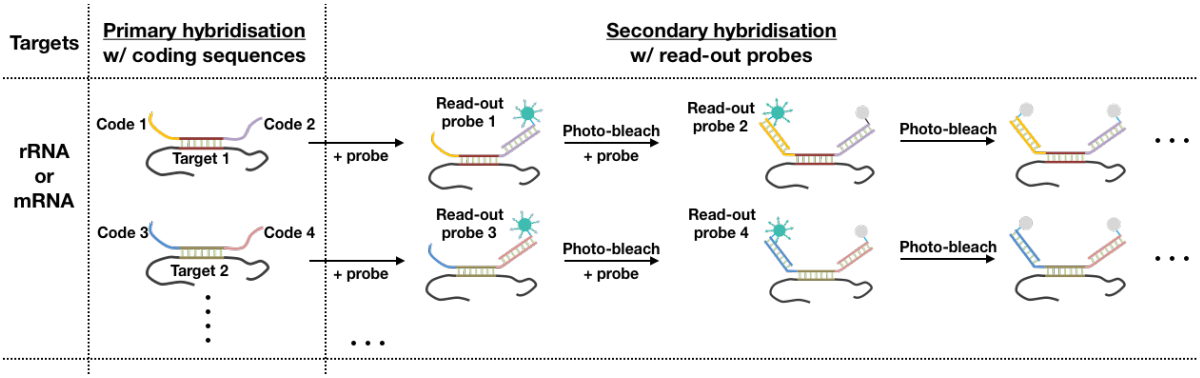

Figure 6 Schematics for a FISH assay with multiple rounds of hybridisation. *The targets can be either rRNA or mRNA. In the first round of hybridisation, all targets are hybridised with their corresponding coding sequences (a long DNA sequence with a target recognition sequence in the middle and two flanking sequences for coding). In the subsequent rounds of hybridisation, a short read-out probe will be added that binds to one code, then photo-bleached, and the next read-out probe will be added, and photo-bleached again.*

Based on MERFISH, the idea of a multicolour coding system is that by using multiple rounds of sequential hybridisation with different coding sequences and readout probes, a large number of targets can be identified. As illustrated by **Figure 7**, in primary hybridisation, N number of long coding sequences bind to specific regions (S_1 – S_N) of the target RNA (S) in the bacteria. Each long coding sequence consists of a 20-nt target recognition part in the centre, flanked by two 20-nt sequences (C_1 – C_{2N}) for coding. The flanking sequences are not complementary to

anywhere on the bacteria genome. In each round of secondary hybridisation, a short read-out probe tagged with a fluorophore, e.g., Cy3 or Cy5, binds to one of the flanking sequences. Once bound with excess probes washed out, it emits fluorescence to indicate success of binding. If not bound, no fluorescence would be detected. Between rounds of adding probes, the whole FOV was photobleached with high intensity of laser. After 2N rounds of secondary hybridisation and image acquisition, a binary coding word can be completed with 1 (green) indicating presence and 2 (grey) indicating absence of a specific region (S). Each binary coding word responds to a target RNA. Therefore, 2N+1 rounds of hybridisation would theoretically differentiate 2^N targets.

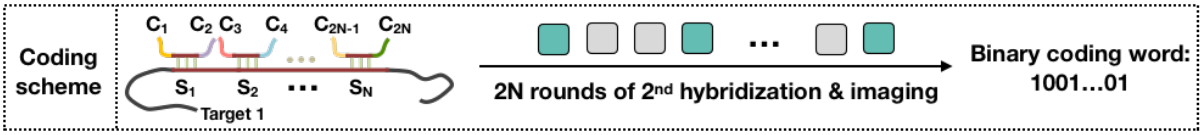

Figure 7 Schematic of a multicolour coding system. *Target RNA (S) has N recognition regions (S_1-S_N). In the primary hybridisation step, each cognition region is bound by one 60-nt long DNA coding sequence. Each coding sequence consists of a 20-nt target recognition part in the centre, flanked by two 20-nt sequences (C1 – C2N) for coding. In each round of secondary hybridisation, one readout probe is added that binds to one flanking sequence (C_1, C_2, …, C_{2N}); if bound, probe will emit fluorescence (shown as a green square and denoted as 1), and otherwise there will be no signal detected (shown as a grey square and denoted as 0). After 2N rounds of secondary hybridisation and imaging, a binary coding word is completed using 1s and 0s, unique to the target RNA.*

2.3 Material and Methods

2.3.1 Cell strains

For the initial development of FISH method, Gram-negative bacteria *Escherichia coli* MG1655 (a non-pathogenic laboratory strain) was chosen as the model organism for its rapid reproduction, ability to survive in various growth conditions, and capability of genetic manipulation. For method validation, two pathogenic clinical isolates, *Escherichia coli* CFT073 and *Klebsiella pneumoniae* MGH78578, were subsequently used.

2.3.2 Cell treatment

Bacteria isolates, both laboratory and clinical, were first streaked on an agar plate and incubated at 37℃. An individual colony was picked from the plate and inoculated in appropriate bacteria growth medium, e.g., lysogeny broth (LB), and then incubated overnight (12-16 hours) in an orbital shaker at 165 rpm at 37℃ Celsius. The overnight culture was diluted 1:100 with the same growth medium and incubated in the same condition. They were harvested when OD_{600} of the culture reached between 0.2 and 0.4 indicating bacteria was in logarithmic growth phase. Cultures were then chemically fixed with 4% (vol/vol) PBS/paraformaldehyde mixture. They were mixed for 20 minutes at room temperature on a nutator. Then washed three times with PBS, each time followed by centrifugation for four minutes at 4000g at room temperature and discarding supernatant and resuspension. Lastly, fixed cells were permeabilised with 50% (vol/vol) ethanol/PBS mixture for 10 minutes at room temperature on a nutator. At this stage, cells could be stored at -20℃ for several weeks.

As clinical bacteria strains were pathogenic, they were chemically fixed in a Biosafety Class-II laboratory in John Radcliffe Hospital with appropriate equipment and safety measures, before bringing back to the Physics Department for further testing.

2.3.3 FISH

To ensure a good quality of the images acquired (approximately 100 dispersed cells in each 13.6μm x 45.5μm field of view), cells were re-suspended well before being added in wells of a gasket on a glass cover slide. The glass cover slide had been coated with either poly-L-lysine (PLL) or chitosan, both of which are positively charged polymers that stick to the negatively charged cell bodies. After 10 minutes of immobilisation, wells were washed three times with PBS. Then coated the empty surface with Calf Thymus DNA to prevent nonspecific binding

of probes to the cover slide surface. Wells were washed three times with PBS before adding probes. To perform hybridisation, probes in hybridisation buffer [0.9M NaCl, 0.02M Tris (pH 7.5), 0.01% SDS, 20% formamide] were incubated with cells for 10 minutes at room temperature in dark, before being washed and imaged.

2.3.4 Microscopy

A three-colour total internal reflection fluorescence (TIRF) microscope was used for imaging. The microscope was designed and built by Dr Peter May, a former member of our research group (**Figure 8**). For probes tagged with Cyanine3 (Cy3), a green laser (532nm) was used for excitation; whereas a red laser (640nm) was used for probes tagged with Cyanine5 (Cy5); power of both lasers was set at 1mW. Images were captured using an electron multiplying charge coupled device (EMCCD) camera. An averaged image over 1000 frames acquired at 30.3ms exposure time is used for each field of view (FOV). All procedures after harvesting cells were carried out in dark at room temperature.

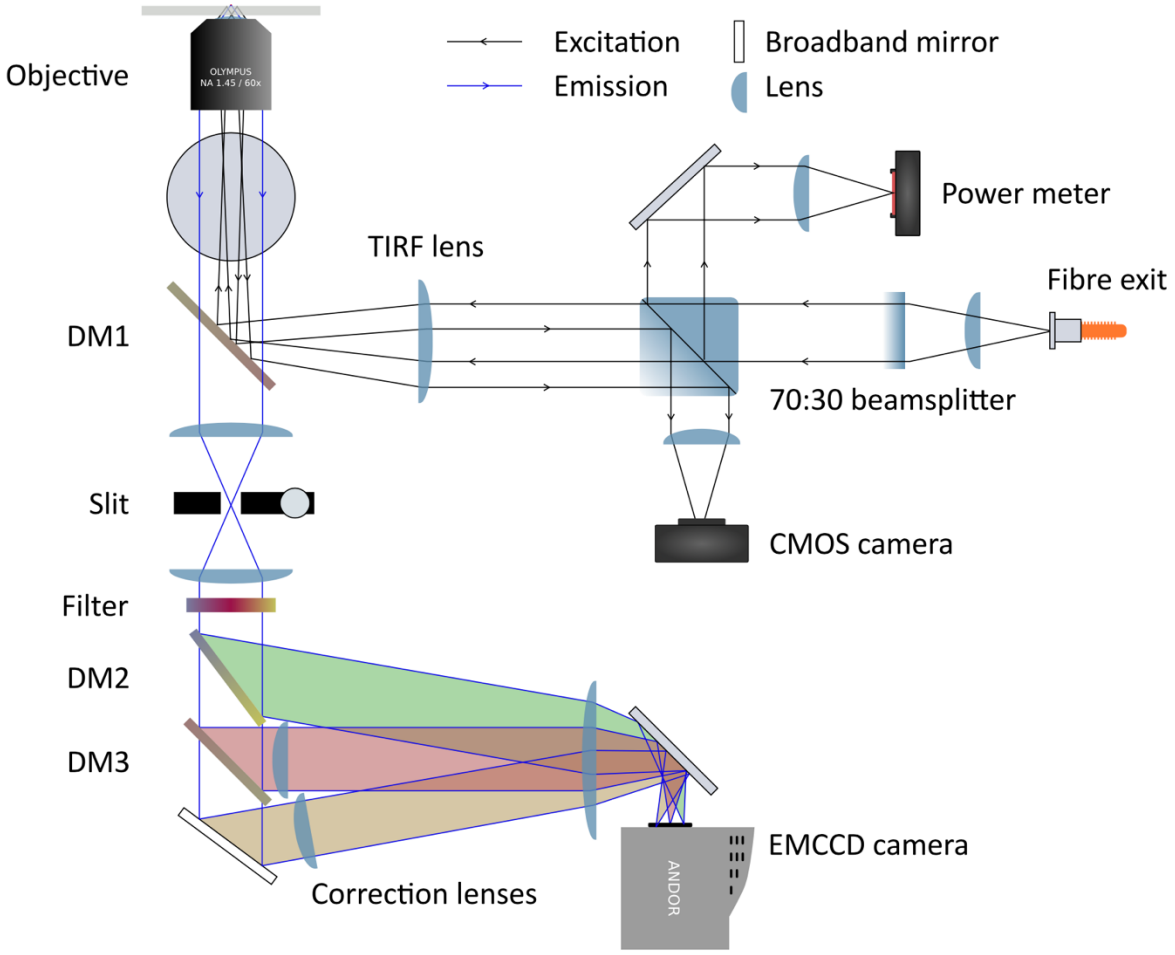

Figure 8 Schematic of the fluorescence microscope. *May, P. (2015) In: Tethered Fluorophore Motion Studies of DNA Segregation Machinery. Figure 5.2, p110. DPhil thesis. University of Oxford, Department of Physics (58).*

2.3.5 Imaging analysis

The images acquired (.tiff files) were stacks of 1000 frames in three channels – green, red, and infrared. First, using ImageJ one image was cut by the channels into three stacks. For each stack a hyperstack was created by averaging the 1000 frames. The dynamic range of all fluorescence images were adjusted to be the same, thus of comparable value. Secondly, using Oufti (an open-source software for imaging analysis) (59), the cells were segmented from the background automatically based on brightfield images. Manual segmentation was sometimes needed when a large number of cells had aggregated and were difficult for the software to segment automatically. Lastly, using MATLAB further image analysis was carried out such as measurement of the average cell intensity.

3 RESULTS

3.1 Development of RNA FISH Assay

3.1.1 Primary hybridisation

To develop the RNA-based FISH assay, the first step was to ensure that primary hybridisation – binding of primary probes to the target – was successful. A rRNA sequence was decided as the detection target, since ribosomes are abundant in a cell and have been used extensively for bacterial identification (54). Specifically, a 20-nucleotide sequence on 16S rRNA, was targeted (**Figure 3**).

To check ability to hybridise, positive control used was EUB_{18}-Cy3, an 18-mer probe tagged with a Cy3 fluorophore on its 5' end. Chemically fixed *E. coli* cells were first immobilised on a glass cover slide inside gasket wells and after 10 minutes of incubation, the unbound cells were washed away. Then probes in hybridisation buffer were added to the cells in wells. After 10 minutes of incubation, the excess probes were washed away. Then the cover slide was mounted on a fluorescence microscopy for imaging.

Upon adding the positive control probes, EUB_{18}-Cy3, to the cells and hybridisation to the targets, Cy3 fluorescence could be clearly detected from all cells as there was an abundance of signal in each individual cell (with an average intensity of 289 arbitrary units, arb. units) (**Figure 9**). All cells were labelled efficiently.

Then a longer DNA was tested that contained a central target recognition and two flanking sequences on the sides. The tested probe was EUB_{58}-Cy5, a 58-mer DNA oligonucleotide with the same 18-nt central recognition sequence as EUB_{18}-Cy3, two 20-nt-long flanking sequences

on the sides and tagged with a Cy5 fluorophore on its 3' end. In the sample shown in the last column, EUB_{58}-Cy5 probes were added; similar to EUB_{18}-Cy3, probes successfully hybridised to the targets and therefore emitted fluorescence (average intensity 168 arb. units).

The detected signals for EUB_{18}-Cy3 and EUB_{58}-Cy5 were distinguishable from the two negative controls: 1) no probe as in the first column, where no probe was added and only very low (101 arb. units) autofluorescence was detected; 2) R1-Cy3 as in the second column. R1-Cy3 was a 20-mer DNA probe tagged with a Cy3 fluorophore on its 5' end. The R1 sequence had no complementarity to the bacteria genome, so it wouldn't be able to anneal anywhere (56). For the sample shown in second column, negative control probe, R1-Cy3, was added, and the same procedure was followed; negligible (111 arb. units) fluorescence was detected showing negligible binding or retention in *E. coli* cells using the protocol above.

Success using a different fluorophore and a longer sequence confirmed the validity of the RNA FISH assay and set the foundation for subsequent rounds of hybridisation.

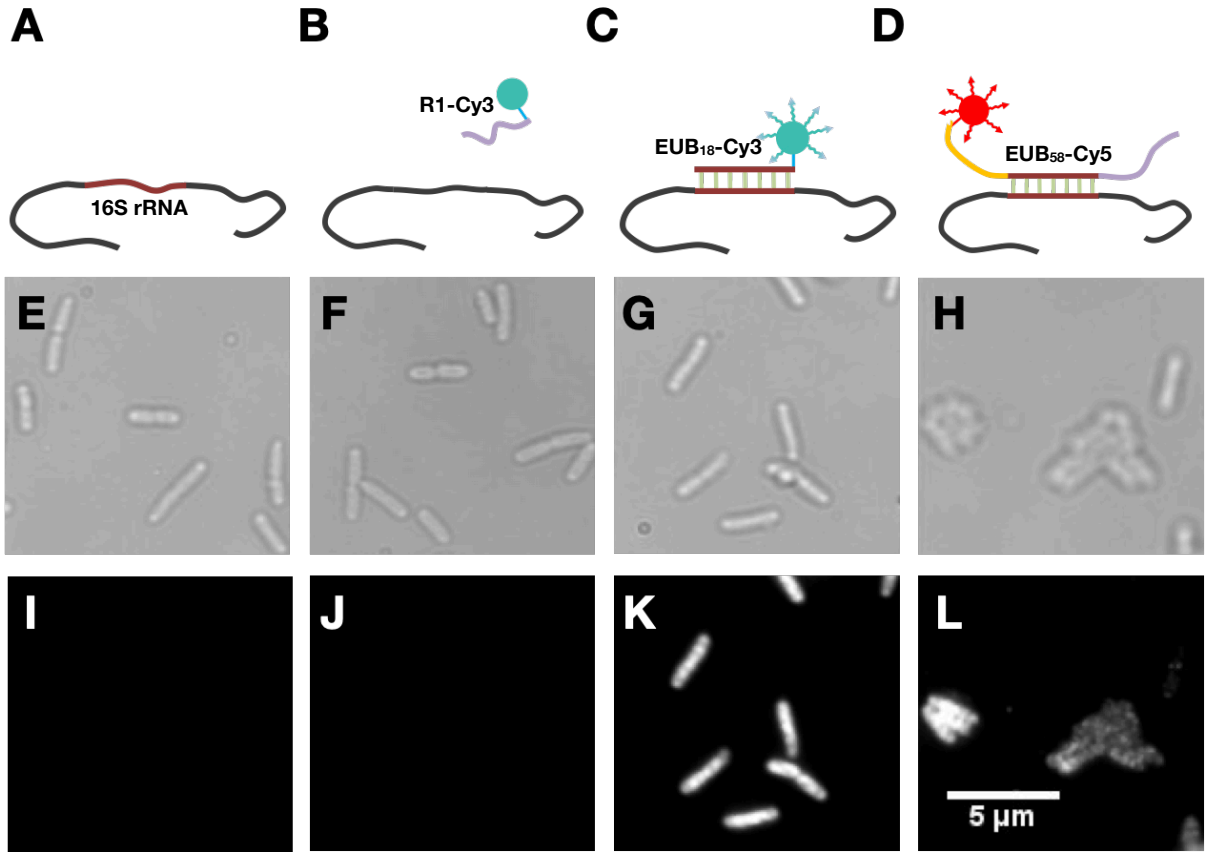

Figure 9 Single-cell FISH experiment – primary hybridisation. *Top row shows the schematics for the sample treatments. Middle and bottom row are brightfield and fluorescence images respectively of the same FOV. Images (12.7µm x 12.7µm) were cropped from a full FOV (13.6µm x 45.5µm). Cells (wildtype MG1655 E. coli) were immobilised on PLL-coated cover slides. In the first column (negative control), no probes were added; In the second column (negative control), R1-Cy3 probes were added which had no complementarity to the bacteria genome; In the third column, cells were hybridised with probes EUB$_{18}$-Cy3; In the last column, cells were hybridised with probes EUB$_{58}$-Cy5. All probes used were at a concentration of 100nM. The fluorescence images were adjusted to the same dynamic range, and there was a clear distinction between successful and unsuccessful primary hybridisation.*

3.1.2 FISH on nucleoid distribution

The fluorescence signals detected in the single-cell FISH experiments were so abundant that the smaller subcellular features couldn't be distinguished. Therefore, a serial dilution of probe concentration was tested on primary hybridisation with the 18-mer Cy3-labelled probes, EUB$_{18}$-Cy3. The concentrations tested were 10pM, 100pM, and 1000pM. As **Figure 10** showed, single molecules could be detected at a concentration of 10pM. The nucleoid distribution observed followed what would be expected from literature on the spatial distribution of

the ribosomes (52), and this showed potential in indirectly reporting AMR-related phenotypes on nucleoid distribution.

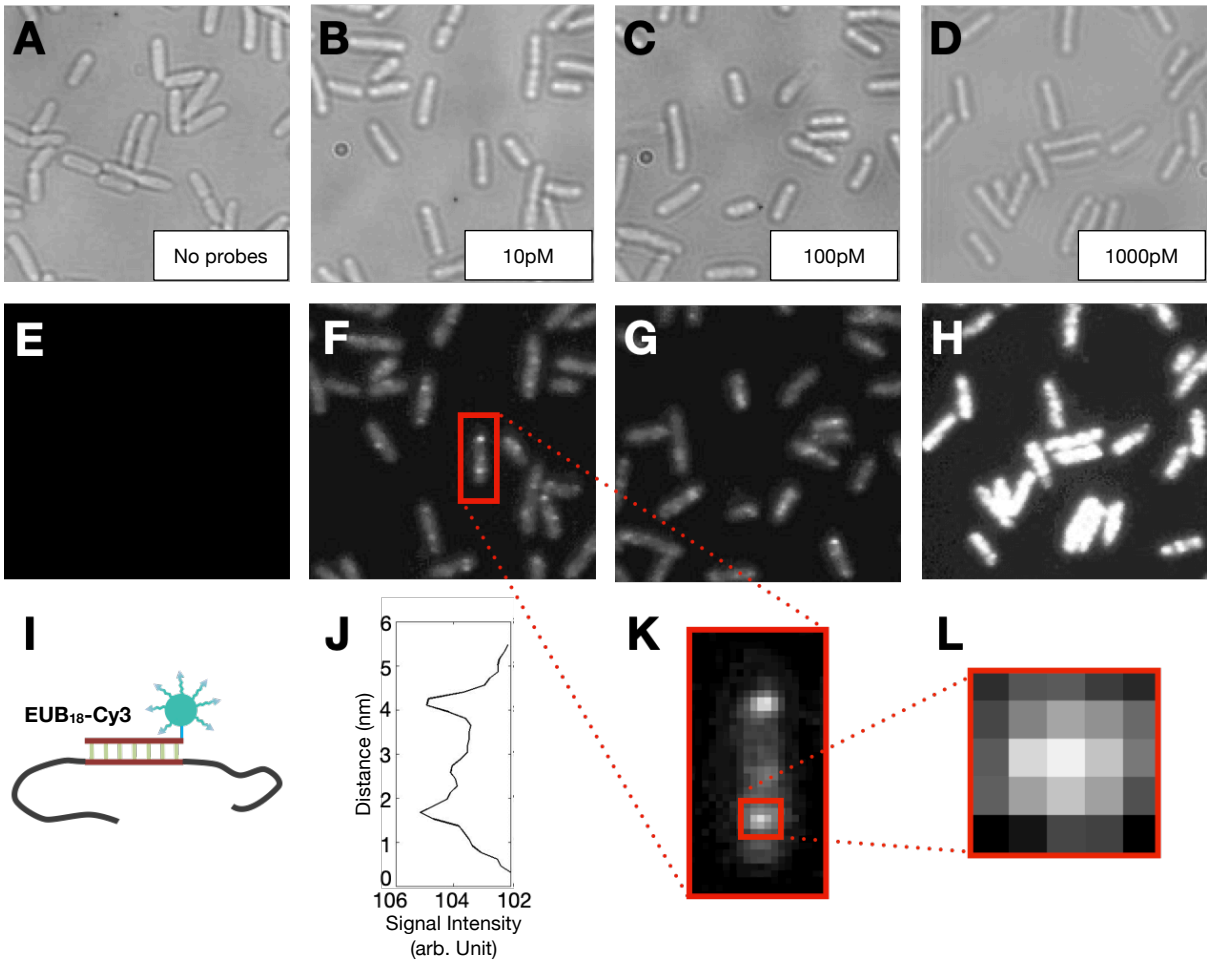

Figure 10 Low probe concentration FISH experiment. *Cells (wildtype MG1655 E. coli) were immobilised on PLL-coated cover slides. In panels A and E, no probes were added; In panels B and F, panels C and G, and panels D and H, cells were hybridised with EUB$_{18}$-Cy3 at 10pM, 100pM, and 1000pM concentrations respectively. Panel I was a schematic of the probe hybridised to the target. Panel J shows a projection along the long axis of one cell (in panel K). Panel L shows signals likely from a group of ribosomes in close proximity.*

3.1.3 Secondary hybridisation

In order to use the sequential approach for bacteria identification, it was essential to ensure that a second hybridisation could be performed after the primary probes were added.

To validate the secondary hybridisation (**Figure 11**), primary hybridisation was first performed using the same procedure described before. Primary probe was a 58-mer that consisted of three parts: the middle sequence recognised and bound to the target RNA, and two flanking

sequences which secondary readout probes would bind. After primary hybridisation was completed with the primary probe, R1-Cy3, a 20-mer Cy3-labelled readout probe, was added. As shown in the fourth column, upon hybridisation to the 3'-end flanking sequence of the primary probe, its Cy3 fluorescence could clearly be detected in all cells with an abundance of signals (average intensity 2372 arb. units).

Similarly, for the sample shown in the third column, R2-Cy3, another 20-mer Cy3-labelled readout probe was added which would bind to the opposite flanking sequence of the primary probe. Upon hybridisation to the 5'-end flanking sequence of the primary probe, its Cy3 fluorescence could also be detected in all cells (average intensity 2355 arb. units).

The detected signals in these two samples were much higher than those detected in the two negative controls: 1) no probes as shown in the first column, where no probes were added and the autofluorescence detected was 100 arb. units; and 2) nonEUB$_{58}$ as shown in the second column, where nonEUB$_{58}$ was used as the primary probe. Probe nonEUB$_{58}$ was a 58-mer sequence similar to EUB$_{58}$ but with the central 18-nt recognition part replaced with its complementary sequence – i.e., it would not bind to the target as they are the same sequence (60). The nonEUB$_{58}$ sequence was also checked and confirmed without self-complementarity. Because nonEUB$_{58}$ probe had been washed away before adding secondary readout probe (R1-Cy3), there was no binding event and thus negligible signal was detected (average intensity 106 arb. units).

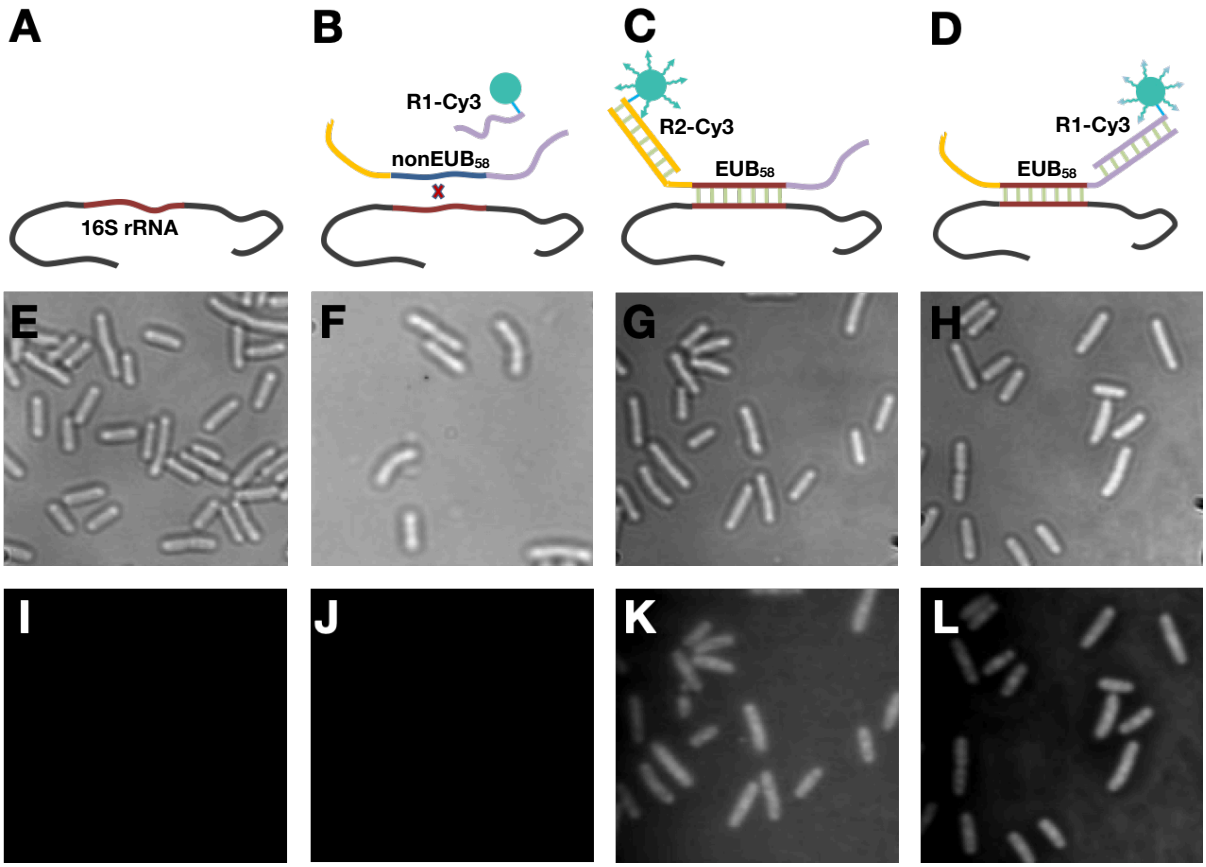

Figure 11 Single-cell FISH experiment – secondary hybridisation. *Cells (wildtype MG1655 E. coli) were immobilised on PLL-coated cover slides. In the first column (negative control), no probes were added; In the second column (negative control), cells were first incubated with nonEUB$_{58}$ with the excess probes washed away, and then incubated with readout probes R1-Cy3; In the third column, cells were first hybridised with EUB$_{58}$ with the excess probes washed away, and then hybridised with readout probes R2-Cy3; In the last column, cells were first hybridised with EUB$_{58}$ with the excess of probes washed away, and then hybridised with readout probes R1-Cy3. All probes used were at a concentration of 30μM. It could be seen that only when the primary hybridisation probes were bound to the target could secondary hybridisation probes subsequently bind and be detected.*

However, a high level of background fluorescence was observed, likely due to non-specific binding of the long primary probes to the cover slide surface. This background fluorescence became much more prominent as the number of targets for secondary hybridisation decreased significantly.

To reduce the background fluorescence, chitosan was used instead of poly-L-lysine (PLL) to coat cover slides and showed significant improvement (**Figure 12**). Over 150 cells were analysed for each coating material, and the differences were consistent. Chitosan is a polysaccharide extracted from shells of crustaceans such as shrimps and crabs. Like PLL, chitosan is also positively charged, making it able to attach negatively charged cell bodies to the surface.

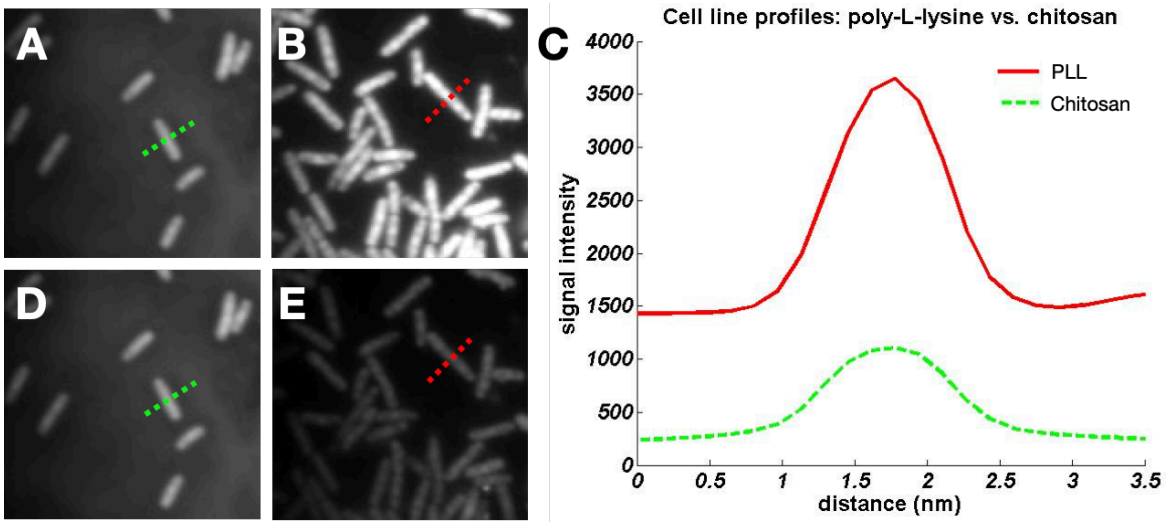

Figure 12 Comparison of image quality using PLL (A and D) and chitosan (B and E) as cover slide coating materials. *Panel D and E have been adjusted to the same dynamic range. Line profiles of cells (C) using two coating materials showed that the background fluorescence detected when using PLL was significantly higher than that when using chitosan. Over 150 cells were analysed with consistent measurements.*

3.1.4 Sequential hybridisation on the same population of cells

After secondary hybridisation was validated, sequential hybridisation was explored where two rounds of secondary readout probes were hybridised and imaged on the same field-of-view (**Figure 13)**.

In this experiment, a cover slide was taped on the translational stage of the microscope after cells were immobilised on its surface. After hybridisation of primary probes to the target rRNA was completed and unbound probes were washed away, a brightfield and a fluorescence image were acquired. Then readout probe, R1-Cy3, was added and upon hybridisation to the primary probes, a fluorescence image of the same FOV was acquired which detected an abundance of signal (average cell intensity 2026 arb. units). Afterwards, this FOV went through photobleaching using 532nm laser at 25mW for 2 minutes. A shorter time of photobleaching could potentially be enough to remove the fluorescence signals to the same extent. Then another readout probe, R2-Cy3, was added and the last image was taken after hybridisation where the fluorescence signal was recovered (average cell intensity 1689 arb. units).

All images had been adjusted to the same dynamic range, and results showed clearly that secondary hybridisation was successful. Bleaching removed 95% of the fluorescence inside cells (down to 103 arb. units) in two minutes and hybridisation of the second readout probes recovered the signals inside cells. This proved that the primary probe EUB_{58} was stably attached and stable to degradation. This provided the proof-of-concept for multiple rounds of sequential hybridisations.

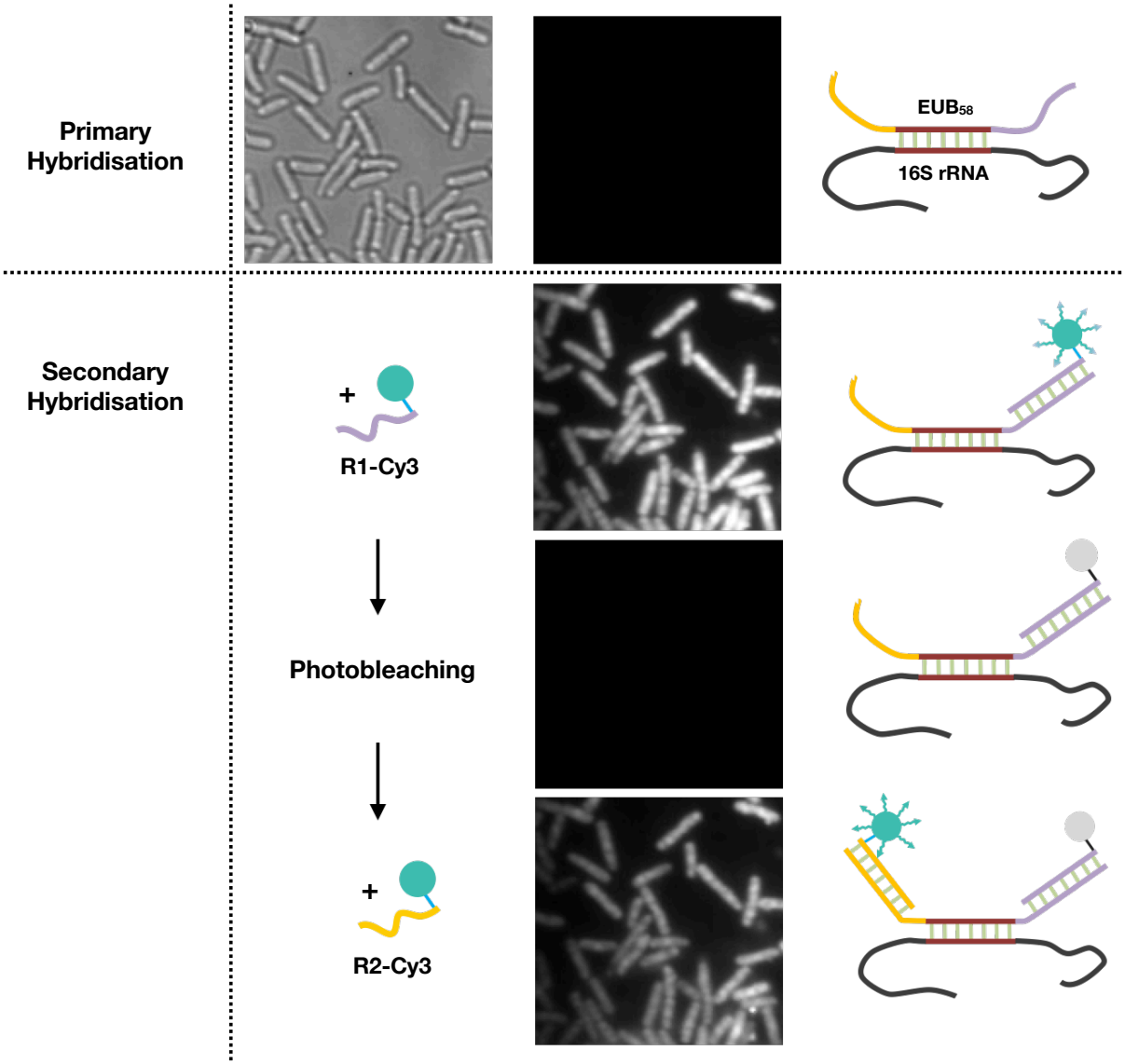

Figure 13 Sequential hybridisation experiment on the same FOV with the same fluorophore. *Cells (wildtype MG1655 E. coli) were immobilised on a chitosan-coated cover slide, which was then taped to the translational stage of the microscope. All probes used were at a concentration of 30μM. Photo-bleaching was performed using 532nm laser at 25mW for two minutes.*

In addition to using readout probes with the same fluorophore, Cy3, sequential hybridisation using different fluorophores, Cy3 and Cy5, was performed and proved successful (**Figure 14**).

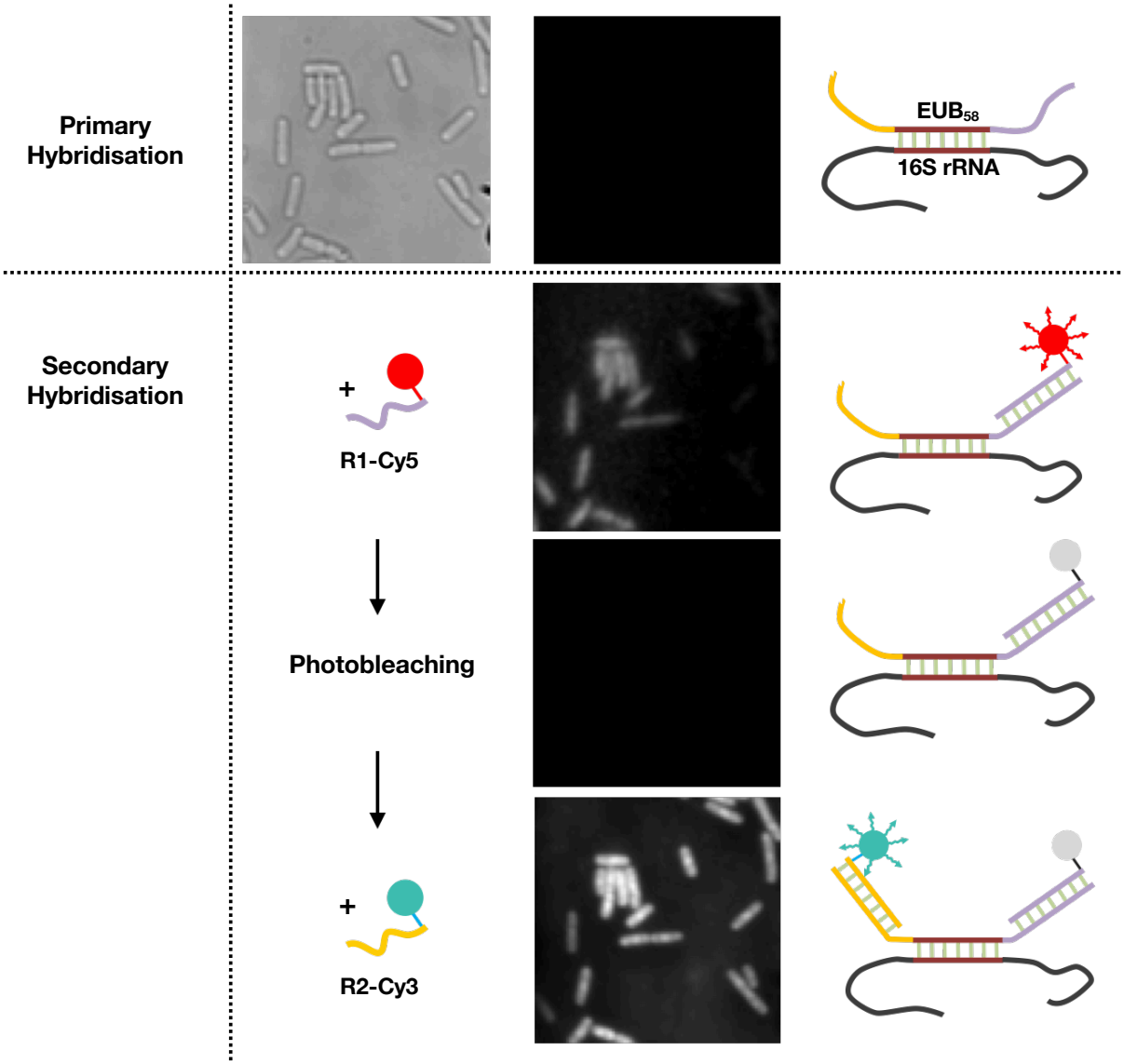

Figure 14 Sequential hybridisation experiment on the same FOV with two different fluorophores. *Cells (wildtype MG1655 E. coli) were immobilised on a chitosan-coated cover slide, which was then taped to the translational stage of the microscope. All probes used were at a concentration of 30μM. Photo-bleaching was performed using 640nm laser at 25mW for two minutes.*

3.2 FISH Targeting 16S rRNA in Clinical Isolates

Following the success of sequential hybridisation, the RNA FISH assay was extended to two clinical isolates: *E. coli* CFT073 – a pathogenic strain isolated from a patient's blood sample in 1989 (61) and *K. pneumoniae* MGH78578 – a pathogenic strain isolated from another patient's sputum sample in 1994 (62) (**Figure 15**). Isolates were first inoculated on commercial

horse blood agar plate, grown in LB, and harvested at OD_{600} = 0.35. Cell treatments prior to hybridisation were carried out in a Biosafety II lab at the John Radcliffe Hospital including fixation and permeabilisation. The chemically fixed cells then underwent the same two-step hybridisation procedure as described before with two different fluorophores and images were taken of the same FOV.

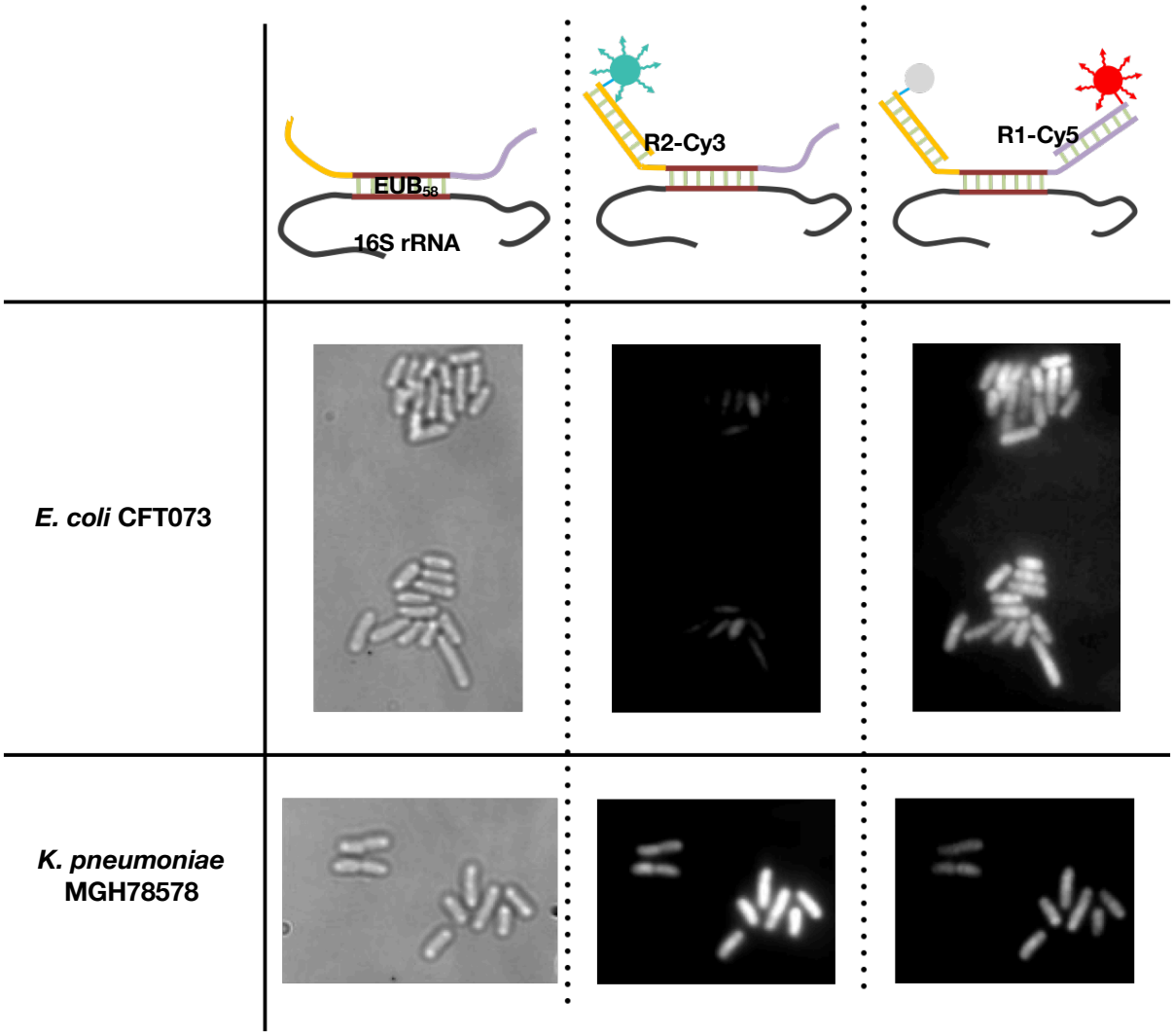

Figure 15 RNA FISH assay tested on two clinical strains (E. coli CFT073 and K. pneumoniae MGH78578) on the same FOV using sequential hybridisation. *Images in the same column has been adjusted to the same dynamic range respectively. Probe concentration was 30uM.*

The aim of this experiment was to confirm that the common FISH probe could detect both pathogens (**Figure 16**). Detection of one readout probe, R1-Cy5, showed no difference between the two cell strains. However, detection of the second readout-probe, R2-Cy3, showed

significant difference between the two strains. Unfortunately, no subsequent trips to the Hospital were made to further explore the discrepancy due to the lockdowns.

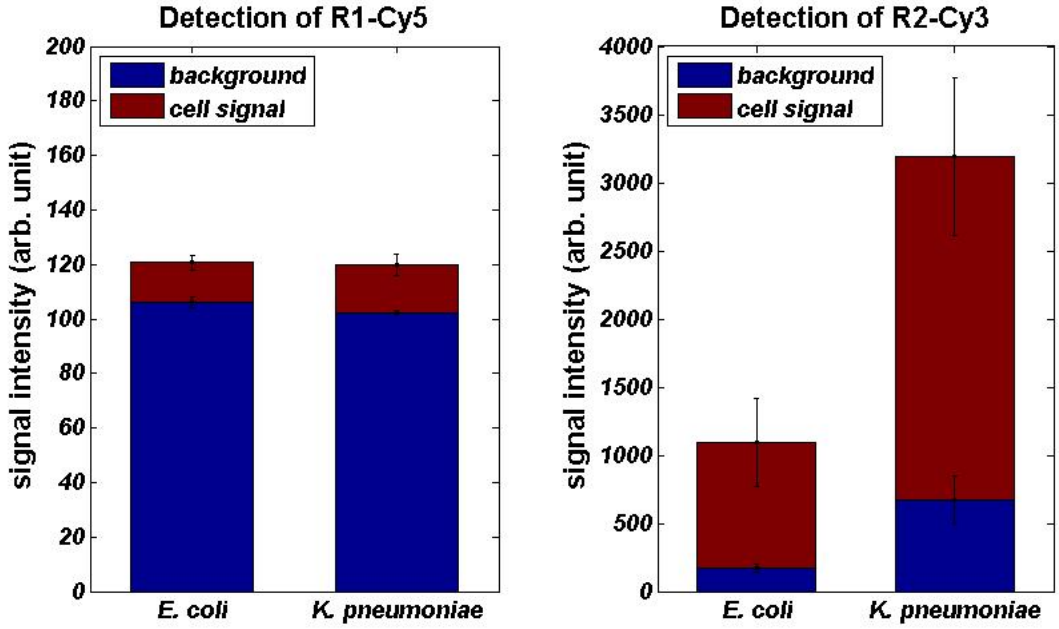

Figure 16 Average signal intensity inside a cell detected in two clinical strains (E. coli CFT073 and K. pneumoniae MGH78578) with respect to two readout probes (R1-Cy5 and R2-Cy3). *Cell counts: E. coli 48 cells, K. pneumoniae 28 cells. Error bar showing standard deviation of the average intensity of all the measured cells and background.*

3.3 FISH Targeting β-lactamase mRNA

While targeting regions on 16S rRNA could lead to bacterial identification, detection of AMR-associated mRNA was still needed to determine whether the bacteria were susceptible to certain antibiotics.

In this experiment, a modified *E. coli* MG1655 strain was used. It contained pUC 19, a high-copy-number cloning vector that carries an ampicillin resistance gene, amp^R (57). The amp^R gene would be transcribed into β-lactamase mRNA, which would then be translated and folded into β-lactamase. β-lactamase hydrolyses the amide bond in β-lactam rings in antibiotics like penicillins (ampicillin is in the penicillin group) and thus providing bacteria resistance against these antibiotics. A new primary probe was designed, Amp_{63}, that targeted a region on the β-

lactamase mRNA. Detection of such mRNA would mean the bacteria possessed and had expressed the resistance gene, thus would confer resistance to ampicillin.

The same experimental procedure was carried out with the new cell strain and the new primary probe (**Figure 17**). From the results we could see that experiment was successful in detecting β-lactamase mRNA. Fluorescence detected from read-out probes on 16S rRNA (second column) and β-lactamase mRNA (third column) clearly differentiated from that in the negative control (first column) where no probes were added.

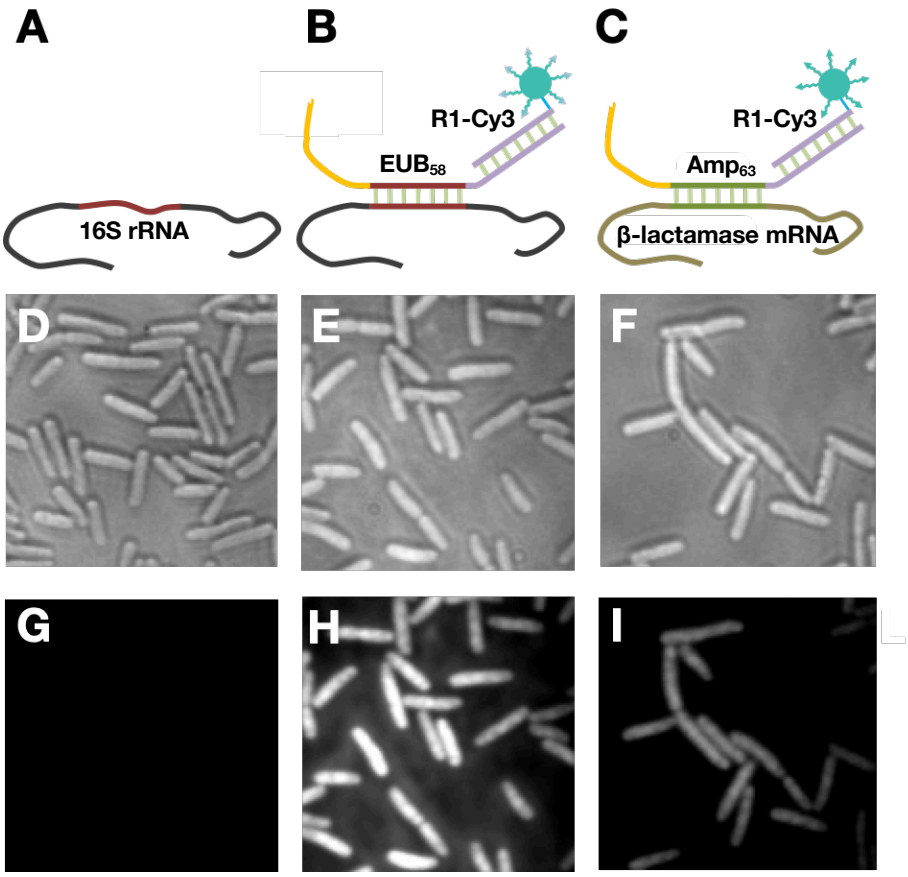

Figure 17 Detection of β-lactamase mRNA in Amp^R cell. *The fluorescence images were adjusted to the same dynamic range. First column (negative control) had no probes added; second column (positive control) detected 16S rRNA; third column detected β-lactamase mRNA, which had a much lower copy number than 16S rRNA.*

The copy number of β-lactamase mRNA in pUC19 strains (500-1000 copies per cell) is at least a magnitude lower than the copy number of 16S rRNA which can reach 55,000 in rich media (57). From the measurements (**Figure 18**) it could be seen that signal detected for β-lactamase

mRNA was indeed significantly lower than that from detection of 16S rRNA. However, the hybridisation efficiency may be different due to different accessibility of the rRNA and mRNA target sequences in the cells. Discrepancy between the ratio of targets' copy numbers and the ratio of signal levels, as well as the potentially different levels of β-lactamase mRNA expression due to various factors would require further investigation.

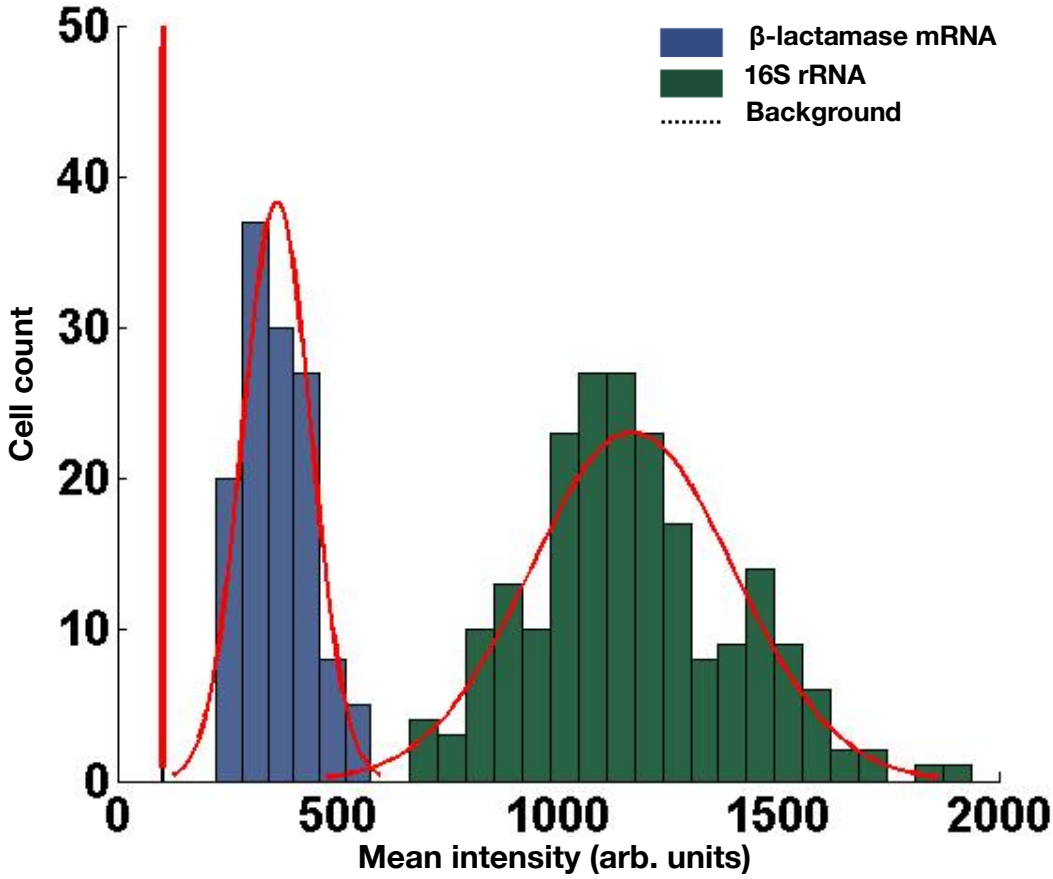

Figure 18 Histograms of signal detection of β-lactamase mRNA and 16S rRNA in Amp^R cells *(Cell count: mRNA 127 cells and rRNA 216 cells). Red lines are the computerised normal distributions of the background fluorescence and signals from cells. The signals of the two targets could clearly be distinguished due to their difference in copy numbers in the cell.*

4 DISCUSSIONS

The goal for this project was to obtain the proof of principle for a rapid imaging assay allowing for both bacterial speciation and diagnosis of the bacteria's susceptibility to a number of common antibiotics. More specifically, an RNA-based FISH assay was developed for this purpose. The assay was conducted on the same population of bacteria using fluorescence microscopy, followed by automated imaging analysis. In this project, only two RNA targets were tested - one region on 16S rRNA and one β-lactamase mRNA.

4.1 Assay Development

The FISH assay was developed and validated step by step, first the primary hybridisation followed by secondary hybridisation, both of which were tested on laboratory strains of *E. coli* to detect 16S rRNA. Subsequently it was validated with pathogenic clinical isolates of *E. coli* and *K. pneumoniae*, and finally the assay was tested on detecting β-lactamase mRNA in a modified strain of *E. coli*.

Several challenges were encountered during the development stage. The biggest challenge was the prominence of non-specific binding of probes to the cover slide surface after introduction of secondary probes. Poly-L-lysine was replaced by chitosan as the coating material of cover slides, and it significantly improved the image quality.

Another notable challenge was that some cells were not completely immobilised or flat on the surface. Some cells would have one end attached to the surface while the

other end was moving in all directions, whereas some other cells would float across the image in and out of focus. The solution was to let the cover slide airdry after washing away unbound cells. Also, the samples shouldn't be left in imaging buffer for too long because it would make cells detach again.

The assay was tested on pathogenic clinical isolates *E. coli* CFT073 and *K. pneumoniae* MGH78578, both of which had been commonly found with multi-drug resistance.

4.2 Merits and Limitations

The RNA FISH assay was able to clearly detect desired targets with excellent signal-to-background ratios. Success of the two-step FISH assay development established the essential foundation for multiple rounds of hybridisation.

A significant reduction in time was achieved compared to other antimicrobial susceptibility tests (AST) as well as conventional FISH, either of which would have taken at least several hours (**Figure 1**). This RNA FISH assay could be completed in under one hour, allowing for fast diagnosis and thus aid in the correct and timely prescription of antibiotics.

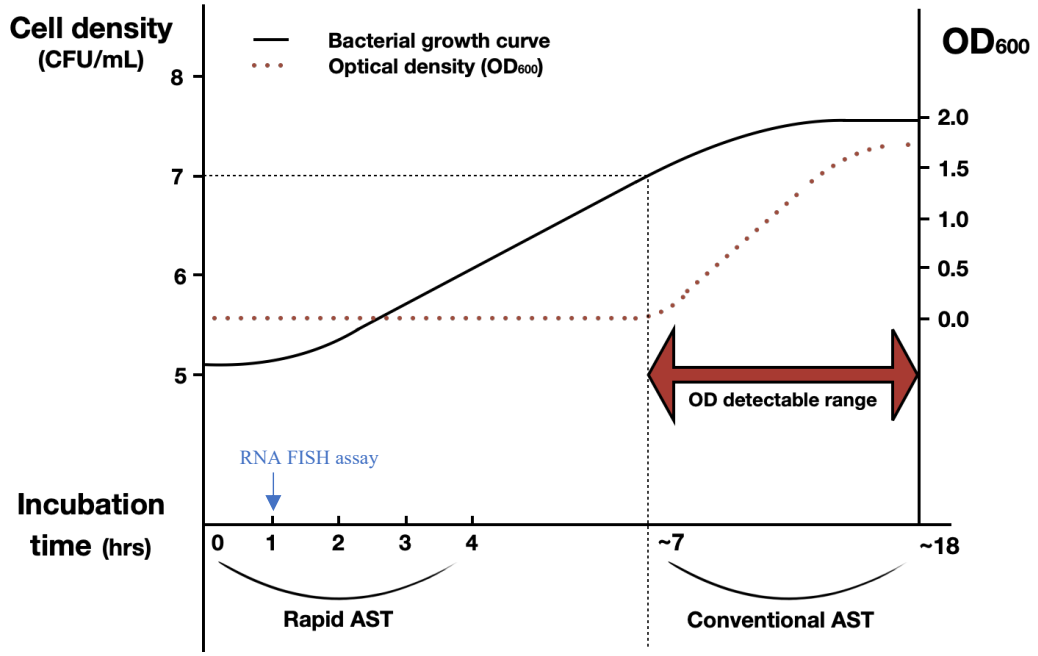

Figure 1 *Bacteria growth curve in comparison with changes in optical density (OD) of liquid cultures for clinical isolates. RNA FISH assay was added to the graph in comparison to other methods.*

One limitation of this RNA FISH assay is that it was rather manual with quite a few rounds of washing and hybridisation. Automation with microfluidics could massively reduce the time needed and the dependence on manual labour. Another limitation is that detecting only AMR mRNAs might not be conclusive whether a certain antibiotic inhibits bacterial growth. This needs to be confirmed with a phenotypic AST that directly detects the antibiotic's inhibitory effect on bacteria growth. Such phenotypic AST is being developed concurrently by other members of the research group.

5 CONCLUSIONS

In this project a proof of principle was successfully developed for a rapid imaging assay for bacteria speciation and genotypic detection of AMR. Compared to 16-24 hours that conventional tests usually take, such assay could be completed under one hour. RNA-based FISH was used for the purpose as it allowed for examination of target genetic materials in their natural environment. Two target RNAs – one region on 16S rRNA and one β-lactamase mRNA – had been examined and all showed clear distinction from negative controls. After the assay was developed, it was validated with clinical pathogenic isolates with promising results.

What has been achieved so far is merely the beginning of a much grander project. The ultimate goal for the entire project is to develop a multiplex imaging assay combining FISH (for identification of bacterial species and AMR mRNAs) and a phenotypic AST. The assay would be automatically performed on a microfluidic device and data analysed all within one hour. Furthermore, the combined assay would be tested on minimally processed clinical specimens in order to be eventually adapted in a clinical setting. In addition to its application in medical diagnostics, this method would also be a versatile tool in a research setting: for studies of other strains of bacteria, as well as studies to look at spatial distribution of transcripts in intact cells, and to look at cellular heterogeneity in cell populations.

One of the immediate next steps is to expand the size of the target library. With the RNA FISH assay already established, it would be able to incorporate more rounds

of hybridisation and thus more targets. The aim is to enable identification of 10 major pathogens based on 16S rRNA and 20 major AMR mRNAs. A foreseeable challenge of signal reduction lies when the copy number of an AMR mRNA is low.

Secondly, the RNA FISH assay would be integrated with microfluidics for automated sample processing. Currently the manual washing of gaskets between steps took the most time, so once operated on a microfluidic device, the procedure could be significantly accelerated.

Thirdly, the RNA FISH assay would be combined with a phenotypic AST. Such phenotypic AST is being developed by another member of the lab, that utilises machine learning to detect morphological changes of the bacteria in presence of antibiotics. The combined assay would ideally be completed within an hour.

Fourthly, the combined assay would be tested on pathogenic clinical isolates. Our clinical collaborators would be providing these strains as well as conducting the standard clinical tests for reference. The isolates would initially be tested individually and then deliberately mixed to see if it could successfully identify different bacteria strains.

Lastly, the combined assay would be tested on clinical specimens provided by collaborators. Same as the clinical isolates, the clinical specimens would go through the standard clinical tests first for reference. A quick but effective cleaning protocol would need to be established on how to handle each specimen (CSF, blood, urine, and pus, etc.) and separate bacteria cells from other human cells and debris.

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