Detecting expression of mRNA of interest in microbial cells *in situ*

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ABSTRACT

We used RNase-H-assisted rolling circle amplification for fluorescence *in situ* hybridization (FISH) to visualize microbes containing transcripts of interest. Our results show that this method is applicable to both Gram-negative and Gram-positive microbes without any noise from DNA and have a potential to quantify the expression of an mRNA of interest at the single-cell level. Therefore, our method can be used as an alternative to single-molecule FISH.

Meta-analyses such as metagenomics and metatranscriptomics are powerful strategies for studying microbiota, including non-cultivable microbes. However, meta-analyses cannot clarify the role these microbes play within microbiota.[1] Although conventional fluorescence *in situ* hybridization (FISH) can be used to identify target microbes within microbiota[2] by probing microbe-specific rRNA, the ecological activities of these microbes cannot be fully elucidated by conventional FISH.[3, 4]

To determine the activities of specific microbes requires the detection of specific mRNAs of interest in the microbiota.[5] In fact, several novel high-sensitivity FISH methods for mRNA detection have been developed using enzymatic signal amplification such as catalyzed reporter deposition-FISH.[6, 7] However, mRNA cannot be quantified by this method, since the signal observed by these methods diffuses over the entire cell. Although single-molecule FISH (smFISH) can detect a single mRNA molecule[8, 9] in microbial cells,[10] this procedure is much more expensive than the other FISH method because it uses nearly 50 different detection probes carrying the same fluorophore against one transcript of interest.[11] A click-amplifying FISH method, termed ClampFISH, was recently developed to amplify the fluorescence signal.[12]

However, this method is time-consuming and confounded by background noise presumably due to nonspecific hybridization of the probe. Most currently available FISH methods, including RollFISH,[13] rely on simple hybridization of oligonucleotide probes to sequences complementary to the probes; thus, these methods cannot clearly distinguish between fluorescent signals originating from RNA and DNA.

FISH using rolling circle amplification (RCA), which uses cDNA transcribed from mRNA by reverse-transcriptase (RT),[14] can detect a single mRNA molecule in a eukaryotic cell. The RCA product (RCP) contains a hyper tandem-repeat of a DNA sequence complementary to a padlock probe (PLP). The RCP eventually becomes a platform on which a sufficient amount of the detection probe can hybridize. Although this procedure is considered to be a substitute for smFISH, it is laborious, requiring an RT reaction, RNase H treatment, and exonuclease treatment. For the detection of microbial mRNA, the exonuclease treatment must be optimized for each individual mRNA under consideration in the operon. For these reasons, a cost-effective and user-friendly FISH method that detects only the target mRNA in a cell is needed.

Recently, we developed RNase H-assisted RCA (RHa-RCA).[15] RNase H digests only the RNA strand of RNA/DNA-hybrid molecules, after which phi29 DNA polymerase can synthesize DNA starting only at the 3′ end of the digested RNA molecules. This procedure allows for the specific detection of almost all types of RNA molecules without using the RT reaction or exonuclease treatment. Here we demonstrate a novel FISH method based on RHa-RCA for the detection of a microbe expressing mRNAs of interest at a single-cell level.

The overall scheme for visualizing a microbe expressing mRNAs of interest by FISH–RHa-RCA consists of five reaction steps (**Fig. 1a**). Using this method, it is possible to complete all steps except fluorescence microscopy in approximately 8 h (**Fig. 1b**). Typically, we perform up to step (iv) on the first day and then store the samples at 4°C, as RCPs are stable at this temperature. On the following day, step (v) and microscopy were performed.

At first, we have tested whether FISH–RHa-RCA detects mRNAs of green fluorescent protein (GFP) expressed in *Escherichia coli* cell. *E. coli* BL21(DE3) transformed with a plasmid carrying GFP gene (pET-AcGFP)[16] was cultured by

inducing GFP expression through the addition of isopropyl-β-d-thiogalactopyranoside (IPTG), whereas the *E. coli* without the addition of IPTG were obtained as negative control (non-induced cell). The non-induced cells were cultured with 2% glucose to inhibit leaky expression of GFP mRNA. The GFP expression was confirmed by fluorescence microscopy using the harvested cells at 2 h after the addition of IPTG **(Supplementary Fig. 1)**.

No fluorescent signal was observed in non-induced cells, even though the cells were also transformed by pET-AcGFP (**Fig. 1c**). In contrast, fluorescent signals were clearly observed in GFP-induced cells (**Fig. 1d**). This result clearly shows that FISH–RHa-RCA specifically detected GFP mRNA molecules and not the GFP DNA in the vector. In addition, the fluorescence signals formed spot-like shapes (**Fig. 1e**), as observed in smFISH.[10, 11, 17] Indeed, FISH using RCA, which uses cDNA transcribed from mRNA by RT, can detect a single mRNA molecule in a eukaryotic cell.[14] Although a eukaryotic cell is large enough to separate the RCPs, bacterial cells are much too small to distinguish each RCPs. Indeed, we observed here that determining the exact number of spots was not possible in the constrained space of these cells.

Therefore, we could not compare the detection efficiency between our method and FISH using RCA after RT. However, taken together, these results suggest that FISH–RHa-RCA may be used as an alternative procedure to smFISH.

Next, we investigated whether FISH–RHa-RCA is applicable to Gram-positive bacteria, which have a thick peptide glycan coat. Using *Brevibacillus choshinensis* harboring a plasmid carrying the DsRed gene under control of the native P2 promoter, we observed fluorescence from the DsRed protein starting at 12 h after the start of the culture. The fluorescence gradually increased, reaching a maximum at 72 h (**Fig. 2, upper images**). Using FISH–RHa-RCA to visualize the DsRed mRNA, the signal in *B. choshinensis* varied greatly between individual cells (**Fig. 2, middle images**). In contrast, the fluorescent signals from GFP mRNA in *E. coli* showed minimal variation between cells (**Fig. 1d**). The fluorescent signal from DsRed mRNA was first observed 12 h after culture initiation, reaching a maximum at 24 h and then decreasing (**Fig. 2, middle images**). The reason the DsRed mRNA expression level varied between individual cells may be that mRNA expression from the P2 promoter occurs during cell wall synthesis, which varies with the cell cycle stage. In addition, it was likely that the

number of signals could be counted in cells with low mRNA abundance (**Fig. 2, lower images**). These results indicate that FISH–RHa-RCA has sufficient potential to quantify the expression of an mRNA of interest at the single-cell level.

Here we report that our method for *in situ* analysis of mRNA in microbes has resolution high enough to detect differences in mRNA expression between individual microbial cells. The use of only one detection probe decreases the cost of our method to approximately 1/50 that of conventional smFISH. In addition, our method produces a stronger signal in a shorter time than does the ClampFISH procedure, because RCP includes a hyper tandem-repeated single-stranded DNA capable of hybridizing a large number of fluorescent probes.

Using RNase H to digest RNA/DNA-hybrid but not DNA/DNA-hybrid molecules, our method can clearly distinguish between mRNA and genomic DNA. Thus, our method does not require use of a PLP at the exon–exon junction to distinguish between them. Thus, we believe that our method for visualizing RNA molecules directly within cells could contribute significantly to both basic and medical research, including the clinical diagnosis of infectious disease.

Ouroboros smFISH was developed recently for examining the expression profiles of numerous genes of interest in tissue sections.[18] Similarly, our method could be upgraded for use in analyzing expression profiles of multiple genes of interest in cells and tissues at a lower cost than Ouroboros smFISH.

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Contributions

H. Takahashi, T. Kobori, Y. Nakashimada, and Y. Okamura conceived of this study and designed the experiments. K. Horio performed nearly all of the experiments. K. Horio and S. Kato collected and analyzed the FISH images. Y. T. Kobori and Okamura obtained the necessary financial support. H. Takahashi, K. Horio, and Y. Okamura prepared the initial draft of the manuscript. T. Kobori, S. Kato, T. Aki, K. Watanabe, and Y. Nakashimada revised the manuscript. All authors contributed to and have approved the final manuscript.

Competing interests

Hiroshima University has filed patent applications related to the technology described in this work to the Japan Patent Office. H.T., K.H., T.A., Y.N., and Y.O. are listed as

inventors on the patents. No one received personal or institutional revenue associated with the patent applications. The JST as the funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. K.H., S.K., K.W., and T.K. declare no potential conflict of interests.

Figure legends

Figure 1. Detection and identification of microbes expressing mRNA of interest by FISH–RHa-RCA. (a) Schematic representation of procedure. (b) Timing and order of FISH–RHa-RCA steps. (c) Detection of GFP mRNA in non-induced *E. coli* cells carrying a GFP expression vector and (d) in GFP-induced cells. Scale bar, 10 µm. (e) Magnified image of box in (d). Scale bar, 5 µm. An overlay of the phase contrast (grayscale) and Alexa-568 labeled probes (red) targeting the RCP from GFP mRNA is shown.

Figure 2. FISH detection of DsRed mRNA in *B. choshinensis* as a Gram-positive bacterium.

Upper images show fluorescence of DsRed protein expressed in *B. choshinensis*; middle images show the signal of detection probes using FISH; lower images show the magnified image of the square box in middle images. The cells in the upper and middle images are not from the same sample because the protein is denatured by the FISH procedure. Overlays of the phase contrast (grayscale), DeRed protein (red), and

Alexa-488 labeled probes (green) targeting the RCP from DsRed mRNA are shown.

Scale bar, 10 μm.

Material and Methods

Padlock probe

Each PLP position is shown in **Supplementary Figs. 3–4**, and the sequences are shown in **Supplementary Table 1**.

Expression of GFP mRNA in *E. coli*

The *E. coli* strain BL21 (DE3) (Novagen) carrying pET-AcGFP was grown in LB medium (1% tryptone, 0.5% yeast extract, 1.0% NaCl, and 50 µg/mL ampicillin) for 14–16 h at 30°C with shaking. The cultures were diluted 1:1000 in fresh LB medium and incubated at 30°C with shaking. Growth was monitored by the measurement of the optical density at 660 nm (OD_{660}). When the OD_{660} was approximately 0.6, GFP expression was induced by the addition of IPTG (final concentration, 0.5 mM). The GFP-induced cells were harvested 1 h after IPTG addition. GFP-induced and non-induced cells were collected in 1.5 mL tubes by centrifugation at 10 000 × *g* for 2 min at 4°C. Cells were immediately suspended in saturated ammonium sulfate (SAS) solution to inhibit RNase activity and store at 4°C before use. This treatment would

keep the total RNA in the cells stable without degradation for at least 2 weeks (**Supplementary Fig. 2)**.

Construction of expression vector for *B. choshinensis* and *in vitro* transcription

The coding sequence of DsRed was isolated from the pDsRed-monomer vector (Clontech) by digestion with the appropriate restriction enzymes. The resulting fragment was cell-free cloned into pNI-His (Takara Bio) for expression in *B. choshinensis* cells as pNI-DsRed or pET-21d (Novagen) for *in vitro* transcription as pET-DsRed. Detailed procedures for cell-free cloning are described in the **Supplementary Material and Methods**.

Expression of DsRed in *B. choshinensis*

The *B. choshinensis* strain HPD31-SP3 (Takara Bio) carrying pNI-DsRed was grown in 2SYF medium (20.0 g/L fructose, 40.0 g/L Phytone Peptone (Becton, Dickinson, and Co.), 5.0 g/L yeast extract (Kyokuto Pharmaceutical Co.), and 0.15 g/L $CaCl_2 \cdot 2H_2O$) containing 50 µg/mL neomycin for 12, 24, 48, or 72 h at 30°C with shaking. DsRed protein expression was confirmed by fluorescence microscopy. The cells were harvested

in 1.5 mL microcentrifuge tubes by centrifugation at 10 000 × *g* for 2 min at 4°C. Cells were suspended in SAS and stored at 4°C before use.

Cell fixation and permeabilization

The stored cells in SAS solution (600 µL) were transferred to a 1.5 mL tube and centrifuged at 20 400 × *g* for 1 min at 4°C to remove the SAS solution. The cell pellet was resuspended in 300 µL of 4% paraformaldehyde and incubated at room temperature for 15 min to fix the cells. After centrifugation, the cell pellet was resuspended with 300 µL of 70% ethanol and incubated at room temperature for 60 min to dehydrate the cells. After centrifugation under the same conditions to remove 70% EtOH, the cell pellet was resuspended with 262.5 µL of 1× TE buffer. Then, 37.5 µL of lysozyme solution (200 µg/mL in 50% glycerol) was added to the cell suspension solution and incubated at room temperature for 10 min to digest the cell wall. After centrifugation at same conditions to remove the lysozyme solution, the cells were washed twice with 300 µL of 1× phosphate-buffered saline (PBS). After centrifugation, the cells were resuspended with 14 µL of µDW and transferred to 0.2 mL of PCR tube to prepare the RCA reaction.

FISH using RHa-RCA

The cells were mixed with 20 pmol of PLP in a buffer containing 20 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate (MgAc), and 50 mM potassium glutamate (KGlu) in a final volume of 20 µL. The PLP was hybridized by incubation at 95°C for 1 min, slowly cooling to 30°C over 30 min, and incubation for 10 min at 30°C. To the cells was added 10 µL of ligation mixture (20 mM Tris-acetate [pH 7.5], 10 mM MgAc, 1.2 mM ATP, 50 mM KGlu, 10 mM dithiothreitol, and 25 units of SplintR ligase), followed by incubation at 37°C for 10 min to seal the PLP. In our previous report,[15] the ligase was inactivated by heating, but this step was omitted in this study to shorten the reaction time and simplify the procedure. The detection sensitivity was not affected (data not shown). The RHa-RCA reaction was started by mixing 30 µL of the ligated mixture with 20 µL of a reaction mixture containing 20 mM Tris-acetate (pH 7.5), 10 mM MgAc, 80 mM ammonium sulfate, 10 mM KGlu, 2.0 mM deoxynucleoside triphosphate, 0.004 units of pyrophosphatase (New England BioLabs), 0.06 units of RNase H (BioAcademia), and 500 ng of DNA-free phi29 DNA polymerase (Kanto

Chemical). The mixture was incubated at 30°C for 2 h followed by enzyme inactivation at 65°C for 10 min.

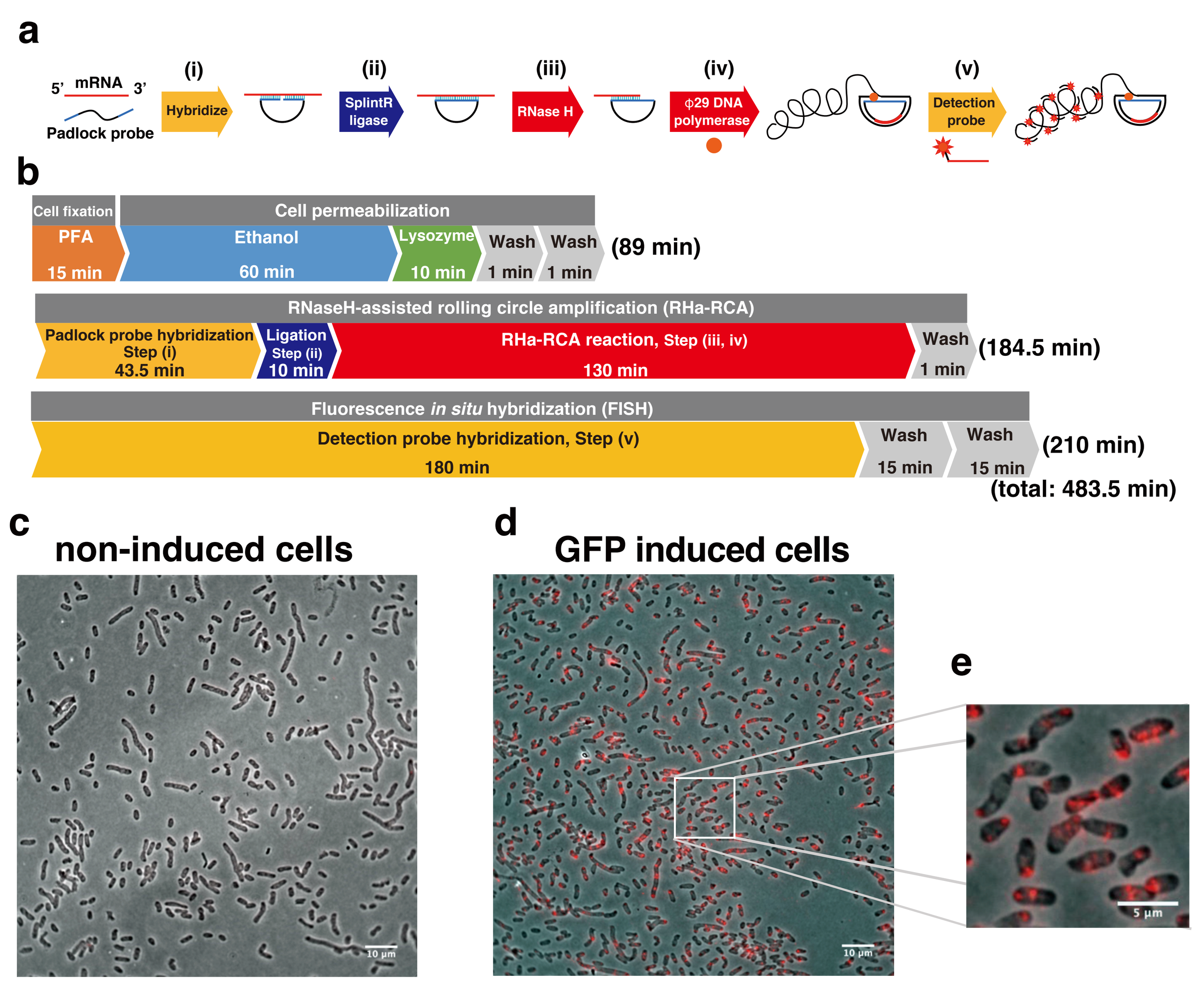
The RCA reaction mixture containing the cells was transferred to a 1.5 mL tube. After centrifugation at 20 400 × *g* for 1 min at 4°C, the cells were washed with 1× PBS at room temperature, suspended in 2× saline sodium citrate, and transferred to a 1.5 mL black tube. After addition of the Alexa-labeled oligonucleotide, the cell suspension was incubated at 37°C for 3 h to hybridize the oligonucleotide to RCP. After centrifugation at 20 400 × *g* for 1 min at 4°C, the cells were washed twice with 1× PBS at 37°C for 15 min. The cells were suspended in 1× PBS and stored at 4°C in 1.5 mL black tubes before observation by microscopy.

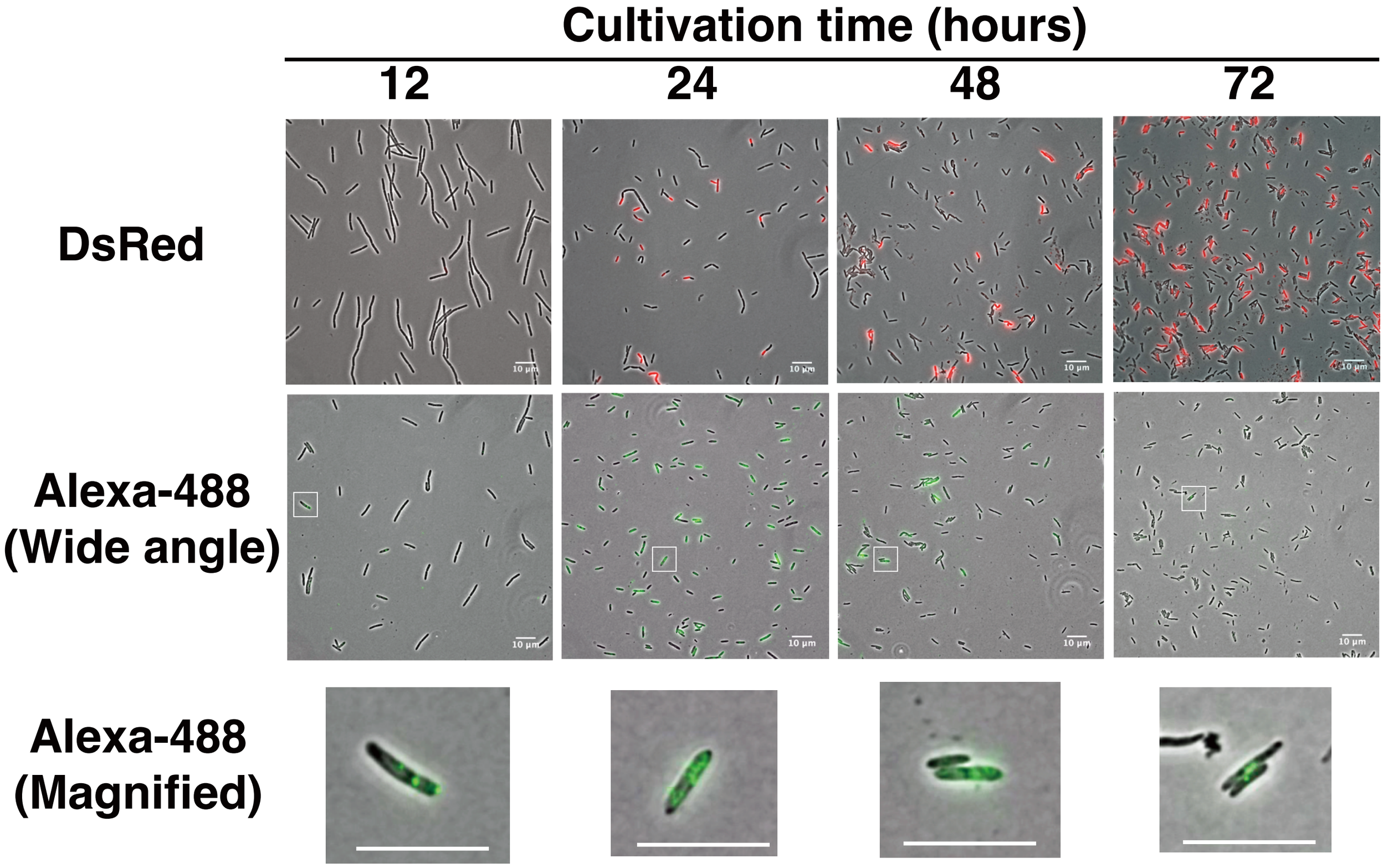
RCA and hybridization were performed in bench-top cleanroom[19] to prevent contamination that could cause nonspecific hybridization..

Imaging and analysis

A drop of cell suspension hybridized with the detection probe was placed on 1% agarose pads containing 1× PBS . Images of each sample were taken on a fluorescence microscope (Nikon ECLIPSE E600 for *E. coli* and Nikon ECLIPSE Ti2-E for

B. choshinensis) equipped with a phase-contrast objective CFI PlanApo DM 100× (Nikon) and an ORCA-Flash4.0 V3 camera (Hamamatsu Co.). Typically, the FISH–RHa-RCA, GFP, and DsRed fluorescence images were taken using an exposure time of 100 msec. The images were analyzed using ImageJ software (NIH).


a
5' mRNA 3'
Padlock probe
(i)
Hybridize
(ii)
SplintR ligase
(iii)
RNase H
(iv)
ϕ29 DNA polymerase
(v)
Detection probe
b
Cell fixation
Cell permeabilization
PFA 15 min
Ethanol 60 min
Lysozyme 10 min
Wash 1 min
Wash 1 min
(89 min)
RNaseH-assisted rolling circle amplification (RHa-RCA)
Padlock probe hybridization Step (i) 43.5 min
Ligation Step (ii) 10 min
RHa-RCA reaction, Step (iii, iv) 130 min
Wash 1 min
(184.5 min)
Fluorescence in situ hybridization (FISH)
Detection probe hybridization, Step (v) 180 min
Wash 15 min
Wash 15 min
(210 min)
(total: 483.5 min)
c
non-induced cells
10 µm
d
GFP induced cells
10 µm
e
5 µm


Cultivation time (hours)
12
24
48
72
DsRed
Alexa-488
(Wide angle)
Alexa-488
(Magnified)
10 µm