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Review Article

From single bacterial cell imaging towards *in vivo* single-molecule biochemistry studies

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Bacteria as single-cell organisms are important model systems to study cellular mechanisms and functions. In recent years and with the help of advanced fluorescence microscopy techniques, immense progress has been made in characterizing and quantifying the behavior of single bacterial cells on the basis of molecular interactions and assemblies in the complex environment of live cultures. Importantly, single-molecule imaging enables the *in vivo* determination of the stoichiometry and molecular architecture of subcellular structures, yielding detailed, quantitative, spatiotemporally resolved molecular maps and unraveling dynamic heterogeneities and subpopulations on the subcellular level. Nevertheless, open challenges remain. Here, we review the past and current status of the field, discuss example applications and give insights into future trends.

Observing biochemistry *in vivo*

Observing the processes of life as they occur in live cells allows us to address questions which we traditionally could not answer by *in vitro* biochemistry experiments using well-defined, but isolated components. Today, many aspects of the behavior of individual molecules in the cellular environment, which in their complexity cannot be reconstituted in laboratory test tubes, become visible through modern optical microscopy techniques.

This ability—to enable *in vivo* biochemistry studies—makes live cell microscopy one of the most powerful tools in today's biological research. The information value of the obtained microscopic images depends on four main factors: *image resolution*, to differentiate between small spatial details within the biological sample, *contrast*, to specifically distinguish between different targets, *sensitivity*, to detect the signal of a single molecule and *temporal resolution*, to observe their (fast) dynamics and interactions. Today, these requirements are met by the various super-resolution fluorescence microscopy methods [1,2].

The recent advances in optical microscopy methods leading to today's sophisticated technologies were possible due to rapid developments in several fields: first, the digital technologies provided new hardware such as highly sensitive and faster cameras and powerful computers as well as new ways of data management and analysis tools, now leading towards AI-assisted and high-throughput super-resolution microscopy [3]. Second, on the biotechnological side, many new genetic manipulation and fast DNA sequencing tools emerged, the latest being the vast gene editing possibilities offered by CRISPR Cas technologies [4]. And finally, the discovery that the green fluorescent protein (GFP) can be used as a general, intrinsic marker for specifically tagging virtually any protein of interest in live cells [5], which sparked the renaissance of fluorescence microscopy. Today the tool box of fluorophore and labeling options to choose from contains a wealth of advanced and specialized fluorescent markers, e.g. biosensors to report on physiological parameters or photoswitchable fluorophores for super-resolution microscopy. With all these technologies at hand, we are now able to design highly filigree and detailed experiments aimed at deciphering the complexity of life *in situ*—from single molecules in individual cells up to whole organisms.
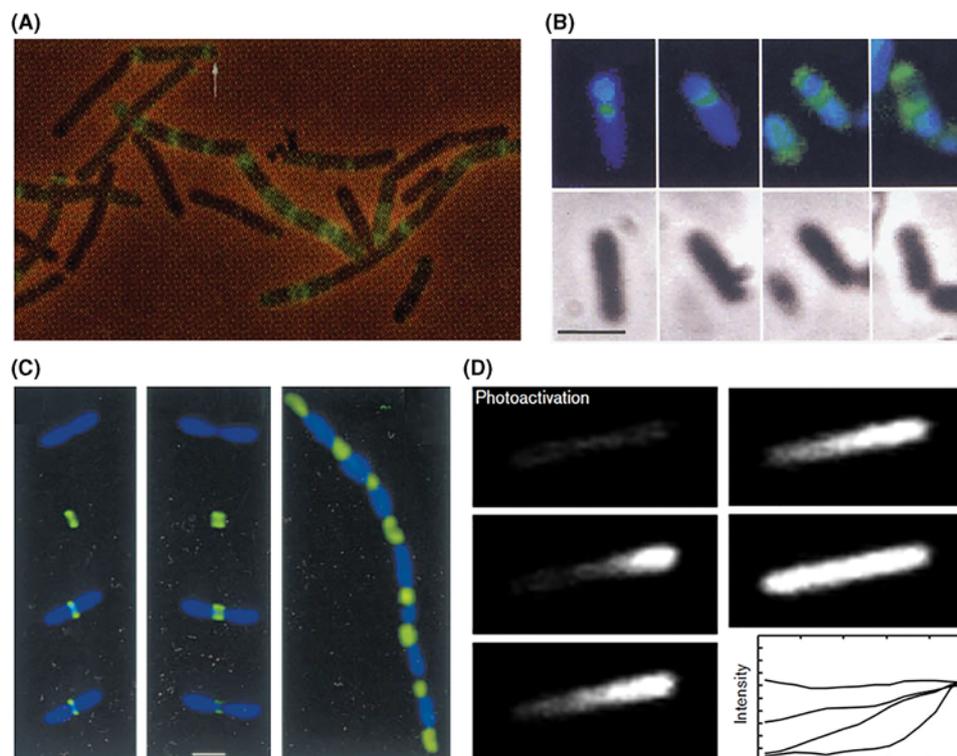
Figure 1. First fluorescence studies using GFP as a genetic marker in bacterial cells
(**A**) Localization of SpoIIE-GFP (green) in sporangia by phase-contrast and fluorescence microscopy at hour 2 of sporulation. Adapted from [6]. (**B**) Subcellular assembly of SpoIVA-GFP-S65T (green) around *Bacillus subtilis* prespores and a filamentous cell, co-stained with DAPI (blue), lower panels phase-contrast images, scale 2 μm. Adapted from [8], original panel letters are removed. (**C**) FtsZ-GFP (green) localizes in rings at mid cell division sites in *Escherichia coli* cells, co-stained with DAPI (blue), scale 1 μm. Adapted from [7], original panel letters are removed. (**D**) Measurement of GFP diffusion in an *E. coli* cell by photoactivation of red-shifted GFP-fluorescence. Snapshots show the cell before (first image) and after (next four images) photoactivation. One-dimensional intensity profiles along the cell for each time step are shown below. Analysis yielded a diffusion coefficient of D = 6.7 $\pm$ 1.6 μm^2/s. Adapted from [11].

The beginnings of *in vivo* fluorescence imaging in microbiology

In microbiology, where the typical sizes of laboratory strains are of the same order of magnitude as the resolution limit of optical microscopy of approximately 200–300 nm (e.g. an *Escherichia coli* cell is approximately 0.5–1 μm wide), the new imaging technologies offered a big opportunity: suddenly, direct live-cell observations (e.g. following the intercellular heterogeneous gene expression patterns of genetically identical bacterial cultures) or subcellular imaging (e.g. following the spatiotemporal organization of specific proteins within individual cells) were possible. It is thus not surprising that the first publication of GFP as a genetic marker lead to an immediate burst of GFP applications localizing various proteins in several bacterial model strains (Figure 1A–C) [6–9]. It was quickly established as a powerful, live-cell compatible alternative to the common immunofluorescence staining of fixed cells [10].

Apart from the spatial distribution of molecules, which demonstrate that bacteria are not just 'bags of enzymes' but indeed maintain a highly organized intracellular organization, also molecular dynamics were addressed. For instance, a pioneering study photoactivated red-shifted GFP at the pole of a bacterium and so attained the first direct diffusion coefficient measurement (in contrast to indirect fluorescence recovery measurements) of a cytosolic protein in bacterial cells (Figure 1D) [11].

The renewed importance of imaging techniques in microbiology approximately 20 years ago can be felt when reading the review of Fung and Theriot from 1998 [12]. It nicely illustrates the times when GFP was new, microscopy went digital and when the concept of super-resolution fluorescence microscopy first emerged.

Subcellular imaging in bacterial cells

At first, subcellular imaging within bacterial cells was focused on imaging clustered molecules at distinct cellular positions, such as the mid cell, the pole or the membrane (e.g. cell shape forming proteins like the actin-homolog MreB [13–15] or the division-ring protein FtsZ [16,17]), or confined in single spots (e.g. specific chromosomal loci [18,19]). Denser spatial organizations like e.g. the cellular distribution of RNA polymerases or ribosomes could not yet be resolved.

For most of these subcellular measurements, proteins of interests were genetically tagged by FPs, e.g. using the bright GFP-variant eYFP [20]. This easy-to-use genetic tagging method is obviously not directly transferable to other cellular components like nucleotides, lipids, or carbohydrates. For those, e.g. when targeting specific chromosomal loci in living cells, several indirect methods were designed: labeling of chromosomal loci was first achieved by using Fluorescence *in situ* hybridization (FISH) assays in fixed cells, visualizing *oriC* and *ter* domains in *Escherichia coli* [21]. For live-cell applications, so-called Fluorescent Reporter–Operator System (FROS) arrays were developed. These systems introduce an array of exogenous DNA sequences, e.g. DNA operator sites, into the genome, which DNA-binding proteins tagged by a FP bind to. For example, multiple copies of the lactose operon were introduced into a chromosomal locus and visualized by GFP fused to the LacI repressor [22,23]. As orthogonal strategies for dual-color imaging, the Tet repressor [24,25] and the parB-*parS* partitioning system [26] were established.

In all cases, the expression of the DNA-binding proteins has to be carefully controlled for: the methods otherwise can lead to a relocation of the tagged loci due to the unusual high protein load or can stall the polymerases replicating or transcribing DNA. The latter effect can of course also be actively used for manipulation studies, e.g. by strategically positioning these arrays as roadblocks for the replication machinery [27]. A similar approach can be used to detect RNA when utilizing FP-labeled MS2 capsid proteins which bind to a specific mRNA hairpin structure [28].

Whereas the first FROS array methods visualizing chromosomal loci in living cells needed multiple copies of the appropriate fluorescent reporters, today individual fluorophores binding to DNA can be detected, e.g. LacI repressors [29] or dCas9 DNA-interference [30]. Furthermore, biosensors like the donor–acceptor pairs for single-molecule Fluorescence Resonance Energy Transfer (smFRET) studies probing the confirmation of DNA can be introduced into live cells by heat shock or electroporation [31–33].

The first detection of an individual FP-labeled protein in live bacteria was accomplished by tracking single copies of a membrane protein, as protein diffusion in the membrane is significantly slower than in the cytosol which simplifies the detection [20]. Faster, cytosolic single proteins were first visualized by directing them to the membrane using an FP tag combined with a membrane-targeted protein fragment [34]. Soon thereafter, utilizing fast stroboscopic illuminations of only 1 ms duration, the first molecular dynamics of cytosolic proteins could be measured [35].

Maintaining and manipulating bacterial cell physiology on the microscope stage

For live cell and especially time-lapse microscopy to yield viable results, it is necessary to maintain the cellular physiology while imaging single cells over extended periods of time. Traditionally, this has been achieved by installing heating on the microscope stages and by constructing so-called agar or agarose pads in which the live cells are embedded in a mixture of low-percentage matrix gels supplemented with growth media. In these pads, cells are hindered from both, passive diffusion and active swimming and thus remain in the microscopic imaging plane but can grow up to several hours before nutrition is depleted. However, despite having been a standard assay for many years, these pads are often not representative of natural environments and cells easily show spatial and temporal heterogeneity and/or compete for resources [36].

Parallel to the microscopic developments, advances in nanofabrication now allow the construction of nano- to micrometer-sized channels and compartments at high precision. These microfluidic devices can be customized in design according to their purpose: most importantly, they allow for the continuous supply of fresh media and nutrients to the sample. However, this active supply can also be used as a reaction chamber to manipulate the cell physiology in real time, e.g. by altering growth conditions changing media or temperature or perturbing the cells with inhibitors, antibiotics or small molecules, e.g. mimicking signaling. This was first achieved by a simple array of linear grooves with a medium flow chamber on top (Figure 2A) [37].

Since these grooves clog up with the growing cells over time, nowadays two different designs are used. One, the so-called chemostat, is built from an array of linear channels with open ends on both sides, where larger, transverse media channels maintain the nutrient supply and wash away overcrowding offspring cells that are pushed out of the channels by culture growth (Figure 2B) [38]. The other, very similar design is the so-called mother machine which is only open to one side (Figure 2C) [39] or possesses an only small outlet for media [40]. This design keeps the mother
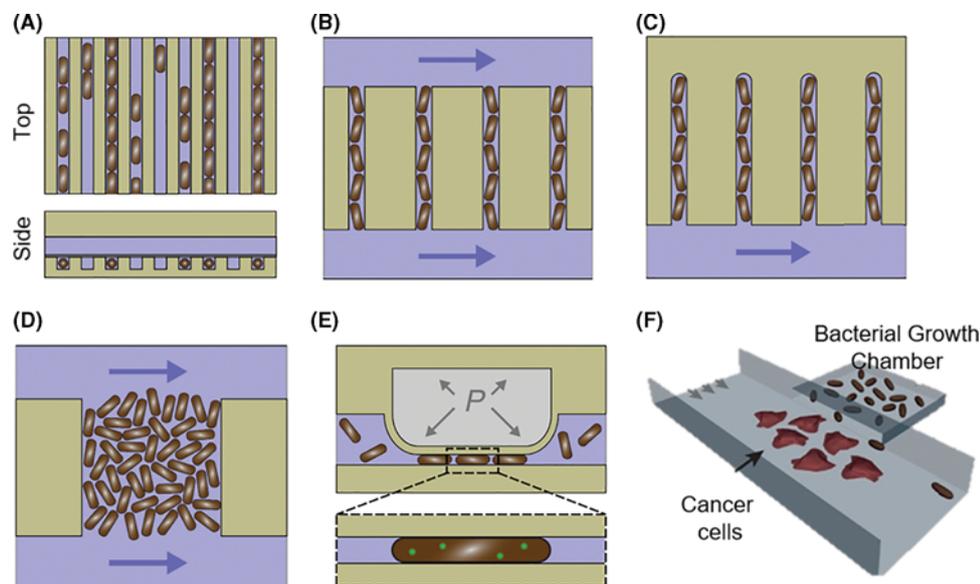
Figure 2. Microfluidic devices for single bacterial cell analyses

(**A**) Array of linear grooves with a medium flow chamber on top [37. (**B**) Array of linear channels with open ends on both sides where larger, transverse media channels maintain the nutrient supply, called chemostat [38]. Overcrowding offspring cells are pushed out of the channels by culture growth and are washed away. (**C**) Similar design to (B) but with only one open side. This design keeps the mother cell in place at the bottom of the channel and therefore produces a linear mini-colony sorted by defined descendance, called the mother machine [39]. (**D**) Two-dimensional monolayer design which ensures direct cell-to-cell contact of larger cell colonies for studying communal behaviors [41]. This device also allows for parallelized, high-throughput measurements. (**E**) Design which can trap and release batches of bacterial cells by applying different pressures on top of the imaging chamber [42]. Additionally, the diffusion of cytosolic molecules in the cells can be slowed down or frozen by this compression. (**F**) Specialized design for following the controlled lysis of bacterial cells into a central channel filled with cancer cells [45]. Schemes (A–E) are adapted from [47], (F) adapted from [45].

cell in place at the bottom of the channel and so produces a linear mini-colony sorted by defined descendance which allows for detailed generation and aging studies, monitoring the emergence of mutations or measuring phenotypic heterogeneity upon antibiotics treatments.

When studying communal behaviors, a two-dimensional monolayer design ensures the direct cell-to-cell contact of larger cell colonies (Figure 2D) [41]. This device also allows for parallelized, high-throughput measurements of several hundreds of cells while still being capable of fast media-supply-switching [29]. A recent microfluidic device can also trap and release batches of bacterial cells by applying different pressures on top of the imaging chamber (Figure 2E). Interestingly, the diffusion of cytosolic molecules in the cells can be controlled very precisely by this compression, slowing it down and even freezing the molecules in place. With this trick, even very low copy numbers (down to two or one) of cytosolic proteins can be counted. It thus makes it possible to quantitate the fluctuation of low molecule numbers which randomize the cellular behavior and cause stochastic heterogeneities in bacterial colonies [42,43]. Finally, specialized devices for such tasks as co-culturing plant roots with bacteria [44] or following the controlled lysis of bacterial cells into a central channel filled with cancer cells (Figure 2F) [45] have been constructed. A more detailed discussion of the different designs can be found in two recent reviews [46,47]. A current challenge is to develop designs which make it possible to select individual cells and to physically isolate them from the chambers. With this at hand, individual phenotypes could be screened for and sequenced—enabling real-time single-cell genetics.

Single-molecule microscopy in bacterial cells

With the advent of super-resolution techniques, not only low copy number molecules can be detected within cells but also moderate to high abundant molecules and dense molecular structures can be resolved. For single-molecule-based super-resolution microscopy methods, this is achieved by fluorescence blinking. This can be implemented either by photophysical or photochemical photoswitching between on- and off-states of the fluorophores themselves as in Photoactivated Localization Microscopy (PALM) [48] and in direct STochastic Optical Reconstruction Microscopy

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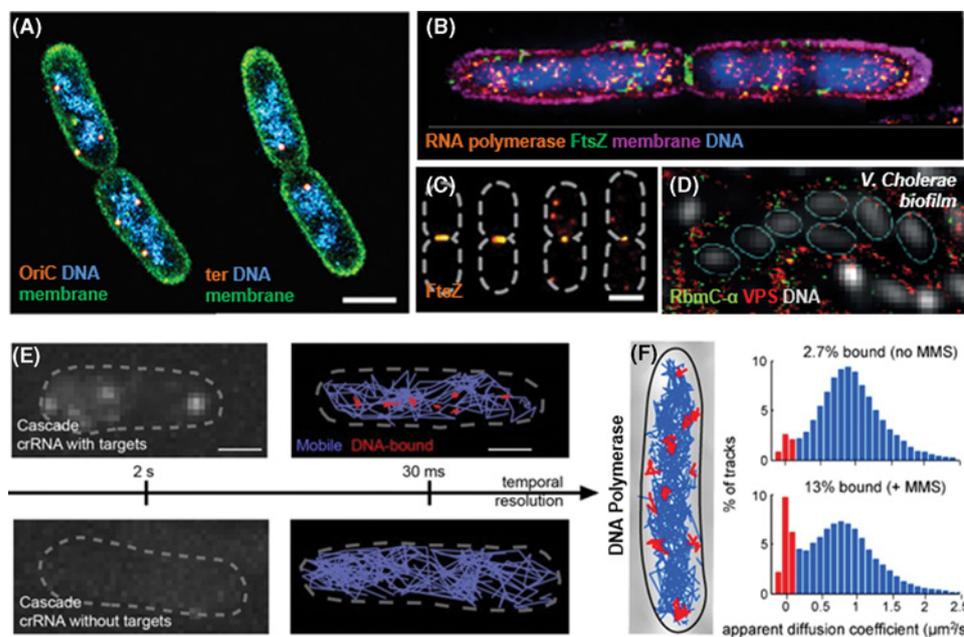
Figure 3. Single-molecule microscopy in bacteria
(**A**) Multi-color image of genetic loci (origin of replication/terminal region, labeled with GFP using a parS-ParB FROS arrays), the nucleoid (JF646-Hoechst) and the membrane (Nile Red) in fixed *E. coli* cells, scale 1 µm. Adapted from [66]. (**B**) Co-localization of the transcription machinery and the nucleoid, co-imaged with FtsZ and the membrane in *E. coli*. Adapted from [93] Copyright CC BY. (**C**) FtsZ division ring in live *E. coli* cells, scale 1 µm. Adapted from [58]. (**D**) Extracellular *Vibrio* polysaccharide (VPS) and protein distributions in live *Vibrio cholerae* biofilms. Adapted from [86]. (**E**) Two *E. coli* strains expressing Cascade complexes carrying different crRNAs. One crRNA is targeted against DNA-sequences in the genome (top panel), the other crRNA has no target sequence present in the host (bottom panel). Imaging with long integration times reveals Cascade complexes stably bound to their genomic targets while unbound Cascades are not detected. Faster imaging rates at higher temporal resolution visualize the DNA-bound and the mobile fractions of transient Cascade interference dynamics, scale 1 µm. Adapted from [82]. (**F**) Measuring single base-excision repair events by DNA polymerase I (Pol1) in live *E. coli*. Pol1 trajectories in a cell in the absence and presence of DNA damage by methyl methanesulfonate (MMS). Histograms of the apparent diffusion coefficient can be used to quantitate the fraction of bound Pol1 molecules, scale 1 µm. Adapted from [100].

(dSTORM) [49,50] techniques, or by reversible on- and off-binding of fluorophores to targets as in Points Accumulation for Imaging in Nanoscale Topography (PAINT) [51] microscopy. By controlling the number of simultaneously active fluorescent emitters, the density of fluorescent spots in a single image can thus be kept at a low, single-spot level and the single-molecule signals can be read-out by sensitive detectors over time by recording an imaging sequence [1,2]. These single-molecule localization data thus enable the *in vivo* determination of the stoichiometry and molecular architecture of subcellular structures such as polymers and multi-protein complexes, yielding detailed quantitative molecular maps of these assemblies and, if combined with single-molecule tracking, quantitative statistical measures of single-molecule processes inside live cells. The resulting massive datasets reveal spatially and temporally resolved molecular maps and unravel dynamic heterogeneities and subpopulations on the subcellular level. Importantly and in contrast to molecular processes measured *in vitro*, these *in vivo* measurements account for the complex environment of cells, e.g. for effects of molecular crowding, different viscosities and charges, potential influences of other cellular machineries or competition of reactions as well as the accessibility of and search times for interaction partners.

Single-molecule microscopy today has been used to measure a wide range of different bacterial cell biology, of which Figure 3 gives a visual impression. It covers research on the cytoskeleton [13,52,53] and the divisome [54–58], the nucleoid organization structure [59–66], replication and repair [67–70], transcription and translation machineries [29,35,71–80], CRISPR Cas interference [30,81,82] as well as secretion systems [83–85] or extracellular polysaccharide and protein distributions in biofilms [86]. A more detailed discussion of these biological studies can be found in

more extensive reviews [87–90]. In short, all studies provide quantitative bionumbers, e.g. reporting on protein numbers, co-localized structures or interaction times, and which can be directly used in biomodeling and simulations. But as impressive as these results look—where are todays technical limits, what are current challenges?

Open challenges in single-molecule microbiology

While single-target studies, mostly using genetic FP tags, are close to getting routine, multi-target or correlative approaches are still rather rare in single-molecule microbiology and need extensive planning [91]. Most available experimental designs are currently dictated by the restricted number of compatible fluorophore and labeling options that fulfill the strict criteria list for high-quality single-molecule microscopy, e.g. yielding satisfactory labeling specificity and efficiency and being read-out at low channel background/cross-talk and at highest spatiotemporal resolution. These requirements make bacterial targets more challenging than mammalian ones, given that microorganisms are protected by robust cell walls, are tightly crowded inside but exhibit still rather low protein abundances in total. Thus, each new technological invention directly feeds back into new possibilities for microbial studies. Current promising developments are, for example brighter, more photostable, fluorogenic or less phototoxic probes [1,66,92–96] or smaller labels allowing for higher labeling efficiencies [97,98]. Also, new imaging techniques promise drastic improvements in temporal resolution and long-term imaging without increased phototoxicity [99]. Nevertheless, how to best visualize interacting and co-moving molecules using single-molecule imaging currently remains an open question [91].

Furthermore, most studies are limited to bacterial model organisms which can be easily cultivated in the laboratory and for which a large number of tools, e.g. genetic manipulation strategies or established sample preparation routines, exist. Applying single-molecule imaging to non-model systems (e.g. medically or ecologically relevant probes such as samples from hospital patients containing pathogens, from the human microbiome, from naturally grown biofilms or from complex co-cultures maintained in bioreactors) is a field which only very scarcely has been explored by advanced fluorescence imaging techniques.

Summary

- Immense progress has been made to study the cellular mechanisms and functions of single bacterial cells in the complex environment of live cultures by microscopy techniques.

- Promising developments were improved fluorescent probes and labels as well as new imaging technologies such as single-molecule microscopy or microfluidic devices to maintain bacterial cell physiology.

- Some open challenges remain, e.g. in the design of suitable multi-target and correlative approaches or adapting single-molecule imaging to non-model systems.

Competing Interests
The author declares that there are no competing interests associated with the manuscript.

Abbreviations
AI, Artificial Intelligence; dSTORM, direct STochastic Optical Reconstruction Microscopy; FISH, Fluorescence *in situ* Hybridization; FP, Fluorescent Protein; FROS, Fluorescent Reporter–Operator System; GFP, Green Fluorescent Protein; PAINT, Point Accumulation for Imaging in Nanoscale Topography; PALM, Photoactivated Localization Microscopy; smFRET, single-molecule Fluorescence Resonance Energy Transfer.

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