Chapter 9
Screening for Antibiotic Activity by Miniaturized Cultivation in Micro-Segmented Flow

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Abstract Despite arduous dedication of the scientific community during the last decades, most screening efforts failed to reveal new antibiotic substances. Droplet-based microfluidics provide a powerful platform to effectively exploit natural metabolic diversity and revitalize the search for new antimicrobials. In this chapter, we explore main requirements to develop and apply droplet-based microfluidic screening strategies for the discovery of novel antibiotics from natural products.

9.1 Introduction: Antibiotics and Antimicrobial Resistance

Short History of Antibiotics and Antimicrobial Resistance: With the discovery of first antibiotics in the 1930s (penicillin, sulfonamides), the eradication of infectious diseases was thought to be just a matter of time. However, even before the introduction of penicillin in 1942, it was discovered that *E. coli* is capable of producing penicillin-hydrolysing β-lactamases, hampering its efficiency as a therapeutic agent [1]. Other molecules representing different compound classes introduced during the "golden era" of antibiotic discovery (1950s) shared a similar fate. Almost simultaneously with their introduction to the market, at least one compound-specific resistance mechanism was unveiled for chloramphenicol, erythromycin or streptomycin, thus challenging the effectiveness of these alleged "silver bullets" [2, 3]. Nonetheless, resistant pathogens were yet not ubiquitous and antibiotics helped save millions of lives threatened by infectious diseases. Since the early 1960s, however, only four new classes of antibiotics were brought to market (Fig. 9.1) [4], while resistant pathogens are becoming increasingly omnipresent. Although time has been gained by chemical tailoring of existing scaffolds, misusage, cross-resistances and a limited number of

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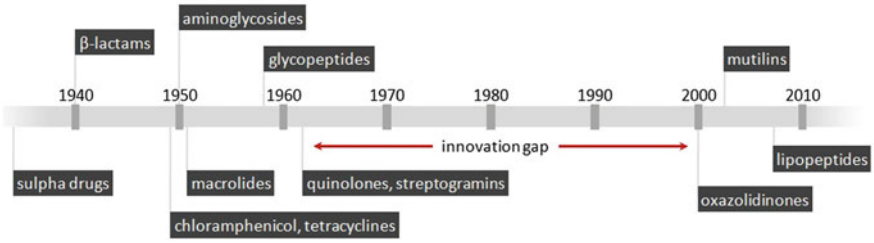

Fig. 9.1 The innovation gap for new classes of antibiotics between 1962 and 2000 (after Fischbach and Walsh [4])

useful derivates constantly diminish this competitive edge. The progressing emergence of new multidrug-resistant (MDR) strains further exacerbates the critical situation, rendering the requirement for new substance classes increasingly urgent [5].

9.2 Current State of Screening for New Antimicrobial Products

Failure of Target-Oriented Screening: After the golden era of antimicrobial compound discovery, classic empirical screening approaches frequently failed to reveal new substances—mainly due to permanent rediscovery of already known compounds. Aside from the carbapenems in 1985, all antibiotics approved for clinical use between the early 1960s and 2000 were synthetic derivates of existing scaffolds [6]. As an attempt to overcome drainage of the lead pipeline, a paradigm shift in screening technology took place: with the dawn of the 1980s, emphasis was put on target-oriented screening approaches, where pathogen-specific putative target-proteins were tested against libraries of natural products as well as libraries of semisynthetic or fully synthetic substances [7]. Despite enormous efforts of pharmaceutical companies dedicated to these strategies, the outcome was disappointing. Although several promising compounds were found, most of them proved to have insufficient membrane permeability [8].

Actinobacteria-Still a Rich Source of New Scaffolds: There is no doubt that the class *Actinobacteria* is still a rich source of new compounds: the theoretical number of substances to be potentially assembled by non-ribosomal peptide synthetases (NRPS) and polyketide synthetases (PKS) is almost infinite. Moreover, there is evidence of a huge pool of so called orphan pathways—gene clusters encoding secondary metabolites not expressed under common cultivation conditions—that remain to be investigated in already known strains [9]. Yet, many of the secondary metabolite producing actinomycetes are still unculturable—often due to inappropriate cultivation methods or low assertiveness of the majority of strains compared to more abundant, fast-growing species like *Streptomyces*. Baltz [10] estimates, that ~99 % of actinomycetes are yet to be discovered. As a consequence, experts propose the return to

discovery of antimicrobial compounds from actinomycetes [4, 11], combined with the achievements of high-throughput-screening (HTS).

High-Throughput Screening of Actinomycetes: Since 1536-well microtiter plates already reach the limit for reliable liquid handling, other technical approaches must be established to maximize throughput [12]. An interesting strategy is pursued by Cubist Pharmaceuticals (Lexington, USA): in a first step, microbial spores from soil samples are encapsulated in 2 mm calcium alginate macrodroplet beads together with culture medium containing antimicrobials to prevent growth of microorganisms other than actinomycetes. After germination and growth, the presence of antibiotics is tested with an *E. coli* strain bearing resistances against the most common broadband antibiotics. Thereby, Cubist Pharmaceuticals is capable of screening 10^7 actinomycetes for antimicrobial activity *per annum*, a magnitude in which the discovery of yet unknown antibiotic producers becomes likely [13].

An alternative approach for high-throughput cultivation of rare species was presented by Akselband et al. in 2006 [117]. Marine bacterial cells were encapsulated in agarose gel-microdroplets (GMDs) of 30-50 μm by bulk emulsification. After incubation, confined microcultures were fluorescently labeled and sorted in a FACS device. Despite enormous HTS-capabilites, this approach does not allow for complex assay regimes, since GMDs and confined microcultures are inaccessible for further assays steps, e.g. addition of a reporter strain.

To overcome these limitations, we discuss biological and technical aspects of bacterial growth in aqueous sub-microliter droplets to establish the detection of antimicrobial activity within microfluidic chip devices. By this, we aim to contribute to a complete whole-cell high-throughput screening for novel antibiotic substance classes from actinobacteria.

9.3 Microbial Assays in Droplet-Based Microfluidic Systems and in Micro-Segmented Flow

9.3.1 General Considerations for Microbial Assays in Droplet-Based Systems

Droplet-based microfluidics provides excellent means to further increase throughput in antimicrobial screening. Figure 9.2 shows a general workflow for detection of antimicrobial activity from a microbial spore suspension. A remarkable feature of this assay is the generation of millions of pure cultures that allow for independent germination and microcolony formation—undisturbed by other highly abundant, fast-growing bacteria [14]. By using the pathogen of interest as a reporter organism, it is ensured that drugs with pathogen-specific activity are selected. This inhibition is then detected as a reduced fluorescent signal from a genetically engineered reporter (e.g. with GFP or derivates). Different approaches are imaginable, mainly determined by the nature of the applied promoter for expression of the respective gene.

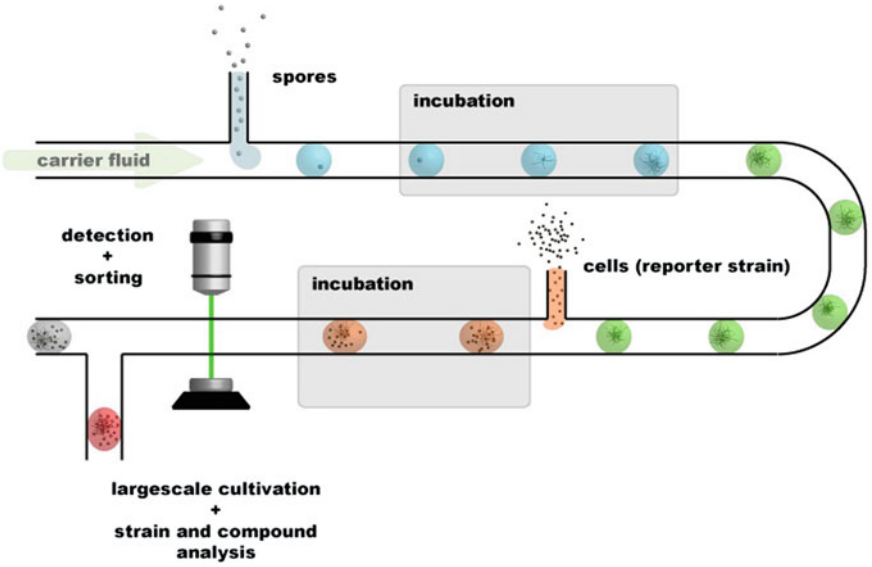

Fig. 9.2 General scheme of screening for antibiotic-producing *Actinobacteria*

Performing whole-cell-assays on a droplet-based microfluidic platform requires a fine tuning of the growth medium regarding optical (background fluorescence, turbidity), rheologic (viscosity, surface tension) [15] and physico-chemical (wettability, critical micellar concentration) demands. In many cases, this translates into suboptimal conditions for bacterial growth with respect to pH-value, mass transfer, and homogenity of substrates and metabolites. Although media droplets can be considered as microscale-bioreactors, they lack common means of process monitoring and regulation, which complicates the optimization of media and strains for an ideal result. In this section, the main requirements to the growth media and reporter strain for cultivation and reliable fluorescence-based detection of antimicrobial activity in droplets are elucidated.

9.3.2 Culture Media for Droplet-Based Screening

In standard laboratory applications, minimal media are frequently used for cultivation of bacteria. Their advantage is the exact nutrient composition, which allows on one hand for high cultivation reproducibility and on the other precise process balancing, which is of great importance in any application where sound understanding of the cell's metabolism is necessary. With regard to actinomycetes, minimal media are traditionally used for basic genetic studies [16, 17]. Moreover, they were employed in studies on RNA polymerases in *Streptomyces coelicolor* [18, 19], in physiological investigations of *Streptomyces viridochromogenes* [20, 21] and for the assessment

of production of antibiotics in actinomycetes [22]. The most prominent minimal medium M9, first referenced by Sambrock et al. [23], contains only mineral salts and 5 g/L glucose.

In contrast to synthetic media, complex broths generally provide better growth conditions and also promote the secondary metabolism of actinomycetes. Hence, with the purpose of finding a suitable culture medium for microfluidic screening of antibiotics-producing actinomycetes, five media with reportedly good promotion of actinomycete growth were selected and optimized towards low phosphate concentration, since it is known that secondary metabolites are often produced at phosphate depletion [24, 25]. An important criterion for exclusion of a broth was the presence of insoluble ingredients, such as oatmeal, soybean meal, corn steep or calcium carbonate, which causes turbidity and hence impedes optical read-out methods to be applied in the final microfluidic assay. 14 different representative actinobacteria were cultivated for 4 days in the chosen media and tested for biomass formation and antimicrobial activity against *E. coli*, *B. subtilis* and *S. cerevisae* in agar diffusion tests (Table 9.1).

Growth was observed in all strain/medium-combinations, and each medium triggered in at least four species production of compounds which inhibited growth of one or more of the reporter strains. Even synthetic M9 medium proved being capable of promoting growth of actinobacteria, but compared to complex media tested in this scope, about 80 % less biomass was produced and inhibition zones were smaller on average, indicating inferior production of antimicrobials.

Modified malt medium ("MMM"), containing malt-, yeast- and beef-extract, appeared to be the most potent. It triggered production of antibiotics in seven out of 14 tested strains, and its supernatants generated the highest number of inhibition zones, i.e. 12 out of 42 possible combinations of actinobacterium and reporter organism. For Boehringer-8 medium similar results were obtained, but the zones of inhibition were in a few cases less distinct compared to the zones of inhibition generated by supernatants of MMM. Due to these findings, MMM was chosen for further experimentation in droplets.

Since active pH-control is not feasible in microfluidic droplets, buffering is the only available strategy to avoid undesired pH-shifts. In the case of MMM (pH 7.2), buffering might have been beneficial for some strains where the pH-value shifted remarkably after 4 days of cultivation. Interestingly, those cultures in MMM where the final pH value was above 7.7 (*K. azatica, A. mediterranei and A. lactamdurans*) or below 5.97 (*N. pusilla* and *S. sibiricum*), the supernatant did not show any antimicrobial activity at all. In case buffering is required for antibiotic screening, it must be desisted from phosphate buffers due to the aforementioned reasons.

Depending on the concentration of complex organic contents (extracts, hydrolysates), the culture media may exhibit particularly high background fluorescence, especially at short wavelength (Fig. 9.3). If fluorescence-based read-out techniques are applied for detection of reporter cell growth or inhibition, strong background fluorescence reduces the sensitivity of the assay. This can be circumvented by dilution of the medium, which might as well be beneficial for the achieved diversity of cultivated strains: it is assumed that less nutritive media, especially with respect to

Table 9.1 Produced biomass (BM [mg]) and inhibition zone diameters (IZ [mm]) for different strain/medium combinations (cultivation: 10 ml in shake flask, 4 days, 28 °C)

Medium		LB			B8			MMM			M9			Glu-So		
Strain		*E*	*S*	*B*	*E*	*S*	*B*	*E*	*S*	*B*	*E*	*S*	*B*	*E*	*S*	*B*
Actinomadura kijaniata IMET 9741	BM	79.4			73.55			45.11			10.19			54.1		
	IZ	0	0	0	0	0	0	0	0	17	0	0	20	0	0	21
Actinoplanes sp. HKI 715	BM	37.22			65.79			22.57			22.91			52.26		
	IZ	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0
Amycolatopsis mediterranei IMET 7651	BM	22.54			37.52			31.77			10.22			80.94		
	IZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amycolatopsis lactamdurans HKI 323	BM	29.96			60.37			37.45			5.19			49.77		
	IZ	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0
Kitasatospora azatica HKI216	BM	36.5			23.44			32.2			10.9			30.87		
	IZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kutzneria viridogrisea HKI 207	BM	41.49			49.78			55.91			11.49			78.2		
	IZ	0	0	0	17	30	17	12	18	2	0	0	10	20	30	22
Nonomureae pusilla IMET 9586	BM	12.05			10.21			22.88			7.9			18.53		
	IZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saccharopolyspora erythrea HKI 184	BM	77.37			73.32			64.43			8.09			54.09		
	IZ	0	0	25	0	0	26	0	0	24	0	0	0	0	0	25
Saccharothrix mutabilis subsp. capreolus HKI 264	BM	50.62			50.81			86.42			16.85			67.39		
	IZ	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0
Streptomyces noursei JA 3890	BM	108.41			59.08			78.92			21.62			54.79		
	IZ	0	30	0	0	60	18	0	50	18	0	33	0	0	11	15
Streptomyces rapamycinicus IMET 43975	BM	66.5			41.9			48.15			8.73			45.63		
	IZ	0	15	15	0	27	14	0	27	15	0	0	15	0	25	11
Streptomyces sp. HKI 714	BM	30.72			30.77			41.88			6.81			48.56		
	IZ	0	25	26	0	23	26	0	26	30	0	0	0	0	25	28
Streptosporangium sibiricum HKI 30	BM	40.21			53.74			71.24			11.98			63.04		
	IZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptosporangium sibiricum FH 8054	BM	77.37			73.32			64.43			8.09			54.09		
	IZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
triggered strains		4/14			7/14			7/14			4/14			6/14		
inhibition events		6/42			12/42			12/42			4/42			11/42		

Antimicrobial activity of supernatants was assessed by agar diffusion tests against *Escherichia coli* (*E*), *Saccharomyces cerevisiae* (*S*) and *Bacillus subtilis* (*B*)

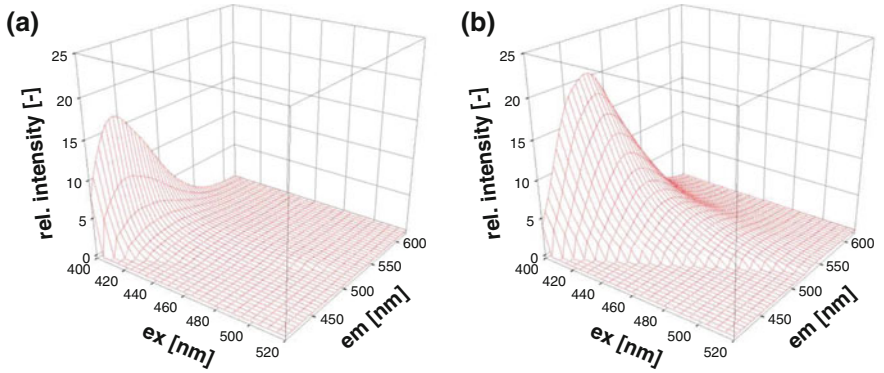

Fig. 9.3 Fluorescence spectrum of M9-medium (**a**) and LB-medium (**b**)

Table 9.2 Organisms and media applied in recent studies in the field of droplet-based microfluidics

Organism	Medium	Modification	Source
U937	RPMI (Invitrogen)	10 % fetal bovine serum	[46]
HEK293T	DMEM (Invitrogen)	10 % fetal bovine serum	[87]
Jurkat	RPMI (Invitrogen)	10 % fetal bovine serum	
S. cerevisae	SD-medium	-	[88]
P. fluorescens, R. rhodochrous, E. coli	LB-medium	-	[59]
E. coli	M9-medium	-	[100]
S. aureus	LB-medium	-	[45]

the carbon source, decelerate the propagation of fast-growing species ("lab weeds") and thus favour slow-growing, less abundant strains [14]. However, the incubation time might increase from days to several weeks.

As shown above, commonly used media often provide a reasonable starting point for tailoring a growth medium according to specific requirements of the cultivated organism and the detection strategy. Table 9.2 lists examples of media employed for the cultivation of cells and microorganisms in recent publications related to droplet-based microfluidics.

9.3.3 Detection Mechanisms for Droplet-Based Screening

The reporting mechanism is the linchpin of every assay. Depending on the application, droplet-based microfluidics provide various means for assessment of ongoing biological, chemical and biochemical processes in single droplets. To maintain droplet integrity and avoid cross-talk between individual droplets, most detection

Table 9.3 Non-invasive readout techniques for microfluidic droplets

	Read-out	Applicability for AB-screening	Reference
Optical	FRET	–	[101]
	Fluorescent proteins	++	[27, 101–103]
	Fluorescent dyes (life/dead staining)	–	
	Fluorogenic reactions	+	[104]
	Luminogenic reactions	(+)	[35, 36]
Non-optical	Amperometric	–	[105]
	Capacitive	–	[63, 106]
	Raman spectroscopy	(+)	[107]

techniques are non-invasive (Table 9.3). For sensing antibiotic-induced inhibition, optical read-outs are the method of choice, since reporter cell-generated signals can be easily tracked and filtered from complex biological and chemical background found in bacterial cultures. Monitoring synthesis of fluorescent proteins (FPs) like GFP, RFP and their derivatives [26] provides a simple means to verify cell fitness. Ideally, measured fluorescence correlates directly with the growth of the reporter strain or with the magnitude of protein synthesis, respectively [27]. Main benefits of FPs include the avoidance of a further assay step in which a fluorogenic substrate is added and their long-term stability. Nevertheless, care must be taken when choosing an FP, since they exhibit great differences in a variety of parameters such as brightness, maturation time and photo stability [28]. In general, derivatives of the wildtype-protein exhibit favourable properties, as for example the "m*Fruit*-series" developed by Shaner et al. [28], derived from the red-fluorescent protein of *Discosoma sp.* Yet, if mass transfer in the incubation device is poor (e. g. in glass chips), maturation and thus fluorescence of the FP can be unsatisfactory due to oxygen limitation [29, 30]. As a work-around, anaerobic fluorescent proteins (e.g. evoglow®, evocatal) can be employed as reporter [31]. However, susceptibility to photo bleaching of such FP should be taken into account, although it might be considered negligible in the case of short exposure times.

Enzymes with a fluorogenic substrate are also generally suited for monitoring cell viability. Baret et al. [32] implemented a β-galactosidase as the reporting enzyme in a highly sensitive fluorescence-activated droplet sorting (FADS) assay, catalyzing the turnover of fluorescein-di-β-D-galactopyranoside to its fluorescent product. Similarly, Agresti et al. [33] established a microfluidic assay to optimize horseradish peroxidase (HRP) itself. Such an approach should also be applicable for an anti-microbial screening assay, using HRP as a representative of entire protein synthesis. Enzymatic assays generally benefit from the conversion of many fluorogenic substrate molecules catalysed by a single enzyme molecule, resulting in leverage of the ratio between synthesized protein to fluorescence. This may be exploited for testing single-cell activity, as already demonstrated for yeast cells [33]. A remarkable drawback of enzyme-based assays is the necessity of rendering the enzyme accessible—either through an additional cell-breakup step (enzymatic lysis [34],

Table 9.4 Fluorescence-based read-out techniques in droplet-based microfluidics

Protein/Enzyme	Substrate	Reference
β-galactosidase	Fluorescein-di-β-D-galactopyranoside (FDG), fluorescein	[104, 108]
PTP1B (target for diabetes mellitus)	Fluorescein diphosphate (FDP)	[104]
HRP	Amplex Ultrared (AUR)	[33]
Arylsulfatase	Fluorescein disulfate, bis(methylphosphonyl)-fluorescein	[34]
YFP (constitutive)	–	[27]
mCherry	–	own unpublished data

electroporation, polymyxin) or by cell surface displaying. Baret et al. [32] relied on a minority of cells in each droplet experiencing autolysis for the above-mentioned FADS assay. In case of an antibiotic screening where co-cultivation of reporter cells and actinobacteria takes place, an increased risk of false negatives is given, since the substrate might not exclusively be hydrolysed by the reporter strain, but also by actinobacteria with yet unknown enzymatic activity. Similarly, live/dead staining, e.g. with resazurin, is also not recommended, for it must be assumed that droplets contain in most cases a living microcolony of actinobacteria, which would frequently cause false positives. Table 9.4 gives an overview of recently applied fluorescence-based assays in the field of droplet microfluidics.

Luminescence-based approaches, such as the firefly luciferase system (lucFF) or the bacterial luciferase derived from *Vibrio fischeri* (luxCDABE), are known to exhibit higher sensitivities and much shorter response times than FP-based assays [35]. Nevertheless, the read-out requires longer integration times, which usually exceeds the droplet's residence time in the measurement section.

Independent of the detection principle, the respective gene must be controlled by a suitable promoter. Three general types of expression control can be taken into consideration: constitutive, compound-inducible and stress-inducible. For several reasons, compound-inducible are preferable to constitutive approaches. First, reporter-cell associated background fluorescence is drastically reduced. This improves the read-out quality and allows for reduction of the droplet volume, which is an important prerequisite for enhancing the throughput of the assay. Accordingly, the inoculation density can be increased, since detection of antimicrobial activity does not merely rely on increment of biomass but also on the degree of protein synthesis. Secondly, taking this concept to its extreme by inoculating with very high cell densities, the time-to-response is reduced: expression and maturation of reporter genes is usually less time-consuming than a sufficient multiplication of biomass. However, if droplets are inoculated with high cell densities, screening is biased towards inhibition of protein synthesis rather than any other mode of antibiotic activity. If detection of the latter is desired, the increment of biomass from an inoculation with low cell densities must be screened. Nevertheless, compound-inducible reporter genes may also

help to reduce the frequency of false negatives in such a case. Finally, addition of an inductor to the spore suspension triggers quasi-constitutive expression once the reporter strain enters the droplet. Yet, most inducers do not interfere or affect the microbiological culture.

Stress-inducible promoters represent an entirely different approach for monitoring antibiotic activity. Depending on their origin, stress promoters are activated upon DNA damage, metabolic changes and quorum sensing related events as well as protein-, DNA-, RNA-, and fatty acid synthesis interference [36]. On the basis of these promoters, sets of antibiotic biosensors have been generated with *B. subtilis* [36] and *E. coli* [37–40] as host organisms. The reporting agent is luciferase in both cases, producing temporary luminescence upon addition of luciferin—its luminogenic substrate. However, the application of stress promoters in HTS for antimicrobial activity is connected to several difficulties: Eltzov et al. reported varying response times for different antibiotics when using the same promoter [39], which results in temporally staggered peaks of reporter activity, and thus variability in the optimal timing for droplet analysis. A further stress-promoter related complication is caused by the missing proportionality of substance toxicity and promoter response. Compounds with strong antimicrobial activity do not obligatorily trigger a vigorous promoter response, while a substance without any pathogen-impeding function might produce a false positive due to promoter activation. In addition, every stress-promoter based assay possesses an upper detection limit set by the minimal inhibition concentration of each combination of antibiotic substance and reporter strain. If this limit is exceeded, the cells might be inhibited or killed before generating a response. Moreover, due to the tendency of stress promoters to leak, the difference between maximal response and background activity (response ratio) is relatively low, impeding reliable detection. In 96- and 384-well microtiter-based assays with *E. coli* biosensors, the highest achieved induction was 255-fold for a combination of a porin related antisense-RNA promoter (micF) and the sulfonamide antibiotic sulfamethoxazole, after 10 h of incubation [39]. The average maximal response ratio of all 14 promoters tested in this study only accounted to 45.3. Also measured in a 384-well microtiter plate, the *B. subtilis* biosensors merely reached a 13.8-fold enhanced luminescence after induction [36]. In contrast, >500-fold increase in total expression is achievable with common compound-induced promoters, e.g. the T7-polymerase system (NEB). However, efforts are being undertaken to enhance response ratio and intensity of stress promoters by sequence-elongation, random mutagenesis, site-directed mutagenesis and promoter duplication [41]. Yet, a striking argument in favor of a stress promoter-based assay is its “inverted” response compared to approaches detecting inhibition of bacterial growth or protein synthesis since screening for fluroescent droplets is technically less demanding than screening for non-fluorescent droplets.

9.3.4 Reporter Organisms for Droplet-Based Screening

Depending on the type of pathogen to be targeted with a potential novel antibiotic, differentiation between three general types of reporter organisms is reasonable: Gram-positive, Gram-negative and fungi. Each type has one or several "work horses" (e.g. *B. subtilis, E. coli* and *S. cerevisiae*) which can also well be utilized in the microfluidic system: they are widely studied, grow fast, produce large amounts of recombinant protein and are flexible with respect to growth conditions, especially regarding C, N and P sources and pH-value. Most importantly, genetic modifications and engineering can be easily performed, due to the manifold of plasmids, kits and protocols that have been tailored for their physiology.

Since filamentous fungi show a tendency to grow out of droplets (personal observation), the group of fungi can solely be represented by yeasts. However, many of today's pathogenic fungi are yeasts (e.g. *Candida* species) and hence worth targeting.

Upon designing a reporter strain, the antibiotic resistances used as selective marker should be carefully taken into account. Discovery of antibiotics belonging to the respective class is ruled out, which might be counterproductive for the actual screening. Yet, rediscovery of already known, abundant classes of antimicrobials is preferably avoided, so that the reporter strain's resistance against this class is beneficial. Cubist Pharmaceuticals took advantage of this screening concept by generating an *E. coli* reporter strain bearing multiple antibiotic resistance genes integrated into the chromosome. This strain was employed for an alginate-bead-based assay for the discovery of novel antibiotics with new modes of action [10].

9.3.5 Aspects of Co-cultivation of Different Microbial Species

After addition of the reporter cells to the droplets bearing actinobacteria microcultures, a co-cultivation takes place, which is related to specific difficulties. Essential nutrients might be depleted due to the growth of actinobacteria and thus not available to the reporter strain. Moreover, as already noted above, media promoting secondary metabolite production of actinobacteria mostly lack phosphate, which is needed for satisfactory growth of reporter strains. Finally, the reporter could be also affected by a shifted pH due to prior cultivation of actinobacteria. Nevertheless, the reporter itself is suspended and dispensed in growth medium, which allows for addition of extra nutrients and buffering agents to the existing droplet. To compensate depleted nutrients, the added medium may also be overconcentrated.

9.4 Detection of Antibiotic Activity in Droplets and Screening for Novel Antibiotics

9.4.1 Possibilities and Constraints of Antibiotic Screening in Droplets

More than 50 % of today's natural products with antibiotic activity derive from microbial secondary metabolites synthesized by actinomycetales [42]. Although approximately 99 % of soil actinobacteria are hitherto still undiscovered, classic, cultivation-based screening approaches fail to reveal new species, mainly due to suppression of less assertive strains and insufficient throughput [10]. According to Watve and Berdy, the decline in discovery of novel antibiotics is not to be ascribed to a decreasing amount of unknown substances rather than a reduction in screening efforts by industries and academia [42, 43]. The microfluidic assay presented here has the potential to revive the interest in antimicrobial screening, since it is cheap (negligible costs for consumables) and fast; thus potentially profitable. Droplet-based microfluidics allows for ultrahigh-throughput generation of pure actinobacteria cultures. Each droplet constitutes a defined nano-scale bioreactor that protects the enclosed bacterial culture against harmful environmental influences, allowing fragile and slow-growing species to develop. The small culture volume also helps to take advantage of intrinsic noise within a species, facilitating detection of antimicrobials that might usually be produced merely in subinhibitory concentrations [44]. Whole-cell-based screening, the most promising approach according to leading experts ([4, 13, 116]), is performed by addition of reporter cells for detection of antimicrobial activity. With this approach, not only target-specificity of a substance is ensured, but also its uptake by the pathogen. However, droplets do not have a solid shell as bioreactors of larger dimensions, which renders the droplet prone to physical and biological influences affecting its size and integrity. Further potential constraints of microbial cultivation in droplets are limited mass transfer and local metabolite accumulation due to missing agitation as well as restricted sensing and control (pO_2, pH, carbon source) (Table 9.9). Moreover, microfluidic channels are subjected to biofouling and clogging, particularly when filamentous organisms as actinomycetes are employed. The following paragraphs elucidate approaches and strategies to assess and possibly resolve all mentioned potential difficulties, and ultimately explore the feasibility of a complete, droplet-implemented screening for antimicrobial substances.

The field of droplet-based microfluidics can be divided into mostly surfactant-free, micro-segmented flow and emulsion-based systems, relying on surfactants. Both approaches are theoretically suited for antibiotic whole-cell-screening and were employed in recent studies related to the detection of antibiotic activity [27, 45–48]. The advantages and disadvantages of each approach will be discussed by means of representative case studies.

9.4.2 Screening for Novel Antibiotics in Micro-Segmented Flow

In general, micro-segmented flow comprises two components: a carrier oil ("continuous phase") and aqueous droplets ("dispersed phase"). As continuous phase, either pure, long-chained carbohydrates (tetradecane, hexadecane, etc.), silicone oils or mineral oils are chosen, depending on the chip material and the nature of its surface. The main criteria for selection of a carrier fluid are immiscibility with the aqueous phase and the prevention of surface-wetting by the dispersed phase, which would result in droplet cross-contamination. Within the experimental setup presented here, tetradecane served as carrier fluid, while the aqueous phase was MMM or LB-medium. Figure 9.4 shows the chip design integrating all required unit operations for antibiotic whole-cell-screening except for final droplet sorting. ~10 nL-droplets are generated from a spore suspension at a frequency of 20 droplets/second, using a T-junction with double-inlet. The dispensing channel for the aqueous phase disembogues in a nozzle shape to provoke interface-tension-dominated droplet tear-off and thus reduce polydispersity at low flow rates [49]. Droplets are stored in the microfluidic chip by stopping the flow after filling the first incubation loop with droplets, spaced by tetradecane. The chip is disconnected, sealed and incubated for several days at 28 °C to promote germination of actinobacteria spores. Subsequently, the chip is reconnected and after restarting the stream of carrier fluid, medium containing reporter cells is added at the second T-junction, simply by applying a continuous flow at a rate that dosage to existing droplets occurs, rather than generation of new droplets (Fig. 9.5). During a second incubation step, now at 37 °C, the reporter cell populations increase in biomass and express the reporting protein. The incubation time is dependent on the applied reporting system, and thus might vary between 2 and 48 h (see Sect. 9.2). The read-out either takes place at halted flow by means of fluorescence microscopy, or is performed in the flowing system with a photomultiplier-based sensor. Sorting of droplets is intended to be performed off-chip.

The double-inlet allows for generation of media gradients upon droplet formation, providing the opportunity to test different growth conditions in a single experiment. Since a serial flow regime is inherent to the presented system, the composition of each droplet can be easily deduced by tracking its position in the series. Consequently, determination of a suitable media composition for optimal promotion of secondary metabolite production in actinobacteria is feasible. This approach can also be applied for activation of orphan pathways unveiled by genome mining [9].

The presented chip displays optimal performance regarding droplet generation: droplets are monodisperse, equally distanced and a lubricant layer at the channel surface is constantly maintained. After 4 days incubation of droplets generated from a suspension containing *S. noursei* spores (10^7 spores/mL $\approx$ 1 spore/droplet), germination and development of typical micropellets could be observed in the majority of droplets. However, distances between the droplets exhibited a much higher variance than upon generation. In rare cases, the inter-droplet distance was reduced to a minimum, which finally led to merging of droplet pairs or even small droplet series. It is known that droplets stored in microchannels move due to fluid-fluid surface tension

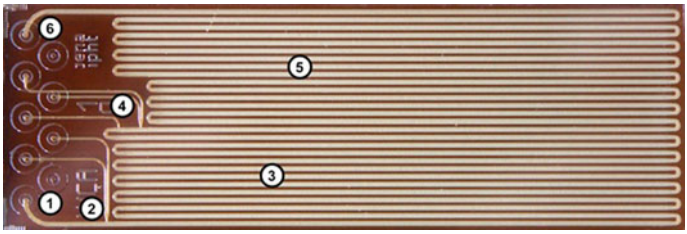

Fig. 9.4 Chip design integrating all structures required for antimicrobial whole-cell-screening. (*1*) Inlet carrier oil (*2*) 2-way droplet generator (*3*) 1st incubation loop (*4*) addition of reporter strain (*5*) 2nd incubation loop (*6*) outlet

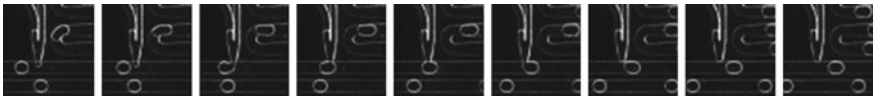

Fig. 9.5 Addition of reporter strain to passing droplet

or solid-liquid surface tension gradients [50]. The first is referred to as "Marangoni convection", caused by temperature (thermo-capillary convection) or chemical gradients along the interphase of two fluids, resulting in a mass flow towards lower interfacial tension. In the system presented here, the probability of chemical gradients leading to local differences in surface tension is high, since microcolonies are not equally distributed over the droplet and metabolites temporally accumulate due to the absence of agitation. Solid-liquid surface tension gradients can either be generated by temperature variations along the channel wall, or by chemical differences—e.g. uneven silanization of the channel surface leading to gradients in hydrophobicity. In both cases, droplets will flow towards higher channel wettability to release interfacial energy.

Upon restarting the flow of carrier oil for addition of reporter cells, maintaining equal distances between the incubated droplets proved to be nearly impossible: while the majority of droplets moved as expected with the stream of carrier oil, some appeared to be stuck in their position, which in consequence led to uncontrollable merging events. A possible explanation for this finding might be a layer of gas bubbles observed on the channel surface, which seemed to hinder droplet displacement. Other plausible reasons might be wetting effects occuring due to settling during long-term incubation or due to the production of biosurfactants [51] that destabilize the lubricant layer. Moreover, since not all droplets contain mycelium, they potentially differ in viscosity, which in turn affects each droplet's velocity to a varying extent [52].

Due to the complexity of the presented assay and the problems inherent to on-chip droplet incubation, the question whether the system is suitable for detection of reporter cell inhibition by an antibiotic has not been answered yet. Droplets generated from antibiotic solutions allow for simulation of in-droplet antibiotic production by actinobacteria, while omitting the most critical step of droplet incubation (including bacterial growth) and thus reducing assay complexity.

To investigate specific reporter cell inhibition in droplets, the whole-cell-assay chip was used for the generation of a droplet series with a gradient in the concentration of the antibiotic nourseothricin—a protein synthesis inhibitor. Without stopping the flow, *E. coli* reporter cells constitutively expressing GFPuv were injected at the intended position (pos. 4 in Fig. 9.4). Subsequently, the droplets were incubated on-chip for 48 h at room temperature (pos. 5 in Fig. 9.4). After the incubation period, image-based fluorescence analyses revealed droplets with regular growth and dark, non-fluorescent droplets where the inoculated cells did not propagate. The clear cut between both droplet species within the series indicated the minimal inhibition concentration of nourseothricin with regard to the employed reporter strain. A Z′-factor of 0.59 was determined from the positive- and negative controls, as a measure for the assay quality [53]. A value of 0.5–1 characterizes an excellent screening.

To go a step further, droplets were generated from culture supernatant of *Streptomyces noursei*, a producer of nourseothricin. This allowed for a more realistic simulation of the in-droplet production of antimicrobial substances by providing the chemical complexity inherent to media in the late phase of cultivation. The concentration of supernatant was altered in a sinusoidal manner along the droplet series. *E. coli* cells producing the red-fluorescent protein mCherry under control of the lac-promoter were injected into the droplets, which already contained IPTG as inducing agent. Again, image analysis displayed inhibition of the reporter by the culture supernatant, allowing for clear determination of a MIC-value. However, the Z′-factor indicated sub-optimal assay quality, probably due to unknown metabolites in the supernatant that affect the behaviour of the reporter cells. Interestingly, besides droplets with "normal" and without cell-derived fluorescence, droplets with strongly enhanced fluorescence were detected, in particular at the transitions between inhibitory and non-inhibitory conditions (Fig. 9.6). This might indicate the occurence of hormesis—i.e. enhanced metabolic activity of microorganisms in the prescence of subinhibitory concentrations of toxic compounds [54, 55]. This effect might be exploited in future screenings: instead of merely picking non-fluorescent droplets (= inhibition) for further investigation, detection and sorting of droplets with significantly enhanced fluorescence could be beneficial, since these potentially indicate the presence of an antibiotic in subinhibitory concentration.

Despite the non-optimal culture conditions to be found in droplets incubated on-chip, particularly with respect to oxygen supply, these results prove the detectability of reporter cell inhibition in the presented chip system.

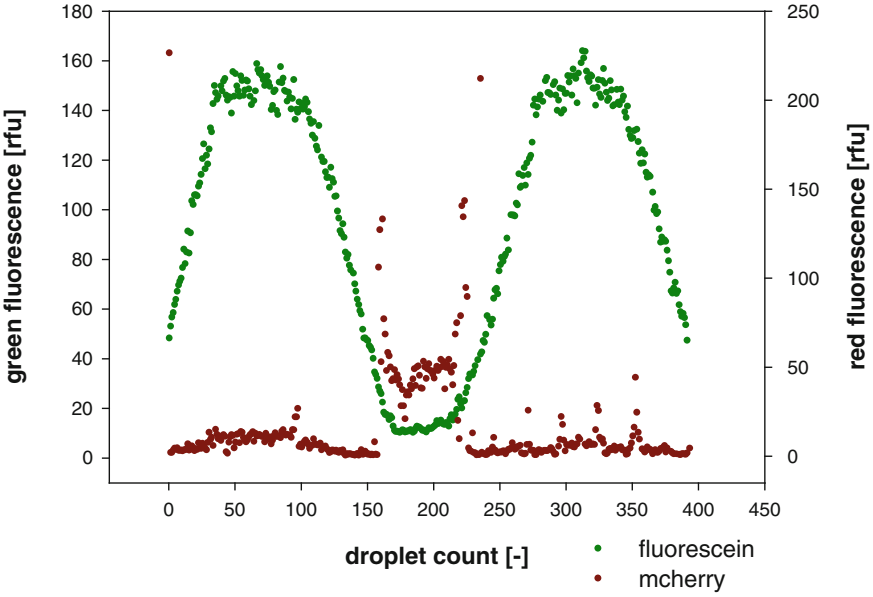

Fig. 9.6 Reporter strain (*red fluorescent*) growth inhibtion in droplets generated with culture supernatant of antibiotic-producing *Streptomyces noursei*. Fluorescein (*green fluorescence*) was used as a marker for supernatant concentration

9.4.3 Improving Robustness of Screening in Micro-Segmented Flow

As indicated above, a complete screening for novel antibiotics from actinobacteria spores with micro-segmented flow is afflicted with substantial problems. However, slight changes in this concept promise improved performance.

Introduction of a third immiscible phase: Instead of separating droplets with carrier fluid, a third immiscible phase may be introduced to the system to avoid contact of aqueous droplets and consequent merging. Zheng and Ismagilov [56] applied this concept in a similar context to test contents of nL-droplets against a large number of reagents (and vice versa). Aqueous plugs were separated by air bubbles, both dispersed in a fluorinated carrier fluid. However, the authors observed carry-over of droplet contents via the dispensing channel. To circumvent undesired cross-contamination, droplets of interest had to be separated by two droplets containing phosphate-buffered saline (Fig. 9.7). In another work by Chen et al. [57] different fluidic systems comprising three liquid phases were developed. The third liquid always served as a separation phase between two reagent plugs. A main challenge was to find combinations of three phases, where engulfment of one dispersed phase into droplets of the second dispersed phase did not occur. To satisfy that condition, the authors predicted that the magnitude of interfacial tension between the carrier fluid and one dispersed phase may not exceed the sum of interfacial tension between

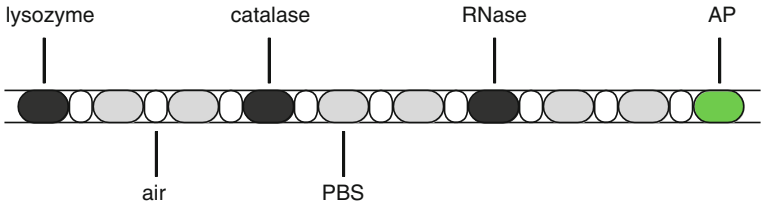

Fig. 9.7 Droplet train separated by air bubbles in a multiple enyzme assay against fluorescein diphosphate. Droplets with enzyme are spaced by droplets containing phosphate-buffer saline (*PBS*) to avoid cross-talk between droplets of interest via the substrate dispensing channel [56]

Table 9.5 Immiscible liquid system comprising three phases with one aqueous dispersed phase

Continuous phase	Dispersed phase (A)	Dispersed phase (B)
FC3283/PFO[a] (10:1 v/v)	1,3-diphenyl-1,1,3,3-tetramethyldisiloxane	water
FC3283/PFO[a] (10:1 v/v)	dimethyl tetrafluorosuccinate	water

[a] 1,1,2,2-tetrahydroperfluorooctanol

carrier fluid and the other dispersed phase plus the interfacial tension between both dispersed phases. These demands were met by two systems comprising aqueous reagent droplets, both with FC3283 as carrier fluid containing 10 % (v/v) PFO as surfactant (Table 9.5). A similar approach was established by Baraban et al. [27]: to provide robustness to a millifluidic droplet analyzer with Novec HFE-7500 (+0.006 % tri-block copolymer surfactant) as continuous phase, aqueous droplets were separated by plugs of mineral oil.

Incubation in tubing: All systems described above, comprising three liquid phases, need at least small amounts of surfactant to avoid wetting of the channel walls by the dispersed phases. Thus, simple dispensing of fluids into existing droplets, as shown in Fig. 9.5 is hampered or even impossible, requiring the integration of electrodes into the chip to destabilize the interphase between carrier oil and aqueous phase by application of an electric field [58]. Of course, the implementation of electrodes increases the demands on chip fabrication and technical periphery. Hence, instead of on-chip incubation of droplets, several authors postulated incubation in PTFE- or FEP tubing, even without use of surfactant or separating droplets of a third immiscible phase: to investigate survival rates of microorganisms in microfluidic droplets after up to 144 h, Martin et al. [59] guided 60-nL-plugs containing different model organisms (*Escherichia coli, Pseudomonas fluorescens, Rhodococcus rhodochrousand Saccharomyces cerevisae*) into teflon tubing of 0.5 mm inner diameter. For analysis, series of 5–30 droplets of the same type were first pooled and then plated out, so that equidistant droplet trains were not as crucial as in an antibiotic screening assay comprising incubation and dosing operations. In follow-up experiments by Grodrian et al. [60], actinobacteria spores were extracted from soil samples, singularized in droplets and incubated at 28 °C for seven days. Again, subsequent analysis was kept simple: to separate droplets for investigation, the capillary was cut into pieces and contents (droplets) unloaded into 100 µl of buffer. Occurrence of any merging events is not reported in this work, implying that it did not appear or only to a negligible extent.

Cao et al. [47] achieved long-term incubation (24 h) of up to 1,100 droplets in PTFE-tube coils for cultivation of *E. coli* in the presence of antibiotic agents. Occurrence of droplet fusion is conceded, but details with regard to frequency are omitted. Nevertheless, the fusion events do not remain unnoticed, since droplet size, -distance and -position are monitored.

Whether external incubation of droplets in tubing and without the help of surfactants leads to increased assay robustness compared to on-chip incubation remains to be elucidated, but in the cases discussed above the authors did not report contrary observations. Since PTFE-tubing does not require silanization prior to usage, a higher chemical homogenity with regard to hydrophobicity of the channel walls might account for minimized droplet movement during incubation.

9.5 Emulsion-Based Microfluidic Screenings: An Overview

9.5.1 Droplet Generation and Handling for Highly Parallelized Operations

The fluidic system for emulsion-based microfluidics generally comprises three components: a carrier fluid, a surfactant (dissolved in the carrier fluid) and the aqueous phase. Due to its amphiphilic nature, the surfactant molecule accumulates at the interphase of dispersed and continuous phase (Fig. 9.8), thus lowering the surface tension of aqueous droplets upon generation. In subsequent steps of the microfluidic assay, droplets may come into contact without the risk of merging due to steric repulsion of surfactant molecules on the droplets' surface. In contrast to surfactant-free systems, "parallel" incubation of droplets in (external) containers is feasible, allowing for immense increase in storage capacity and thus throughput. At the same time, the robustness is enhanced, since maintaining equidistant droplets is no longer necessary.

Various recent publications demonstrate the enormous potential of surfactant -stabilized, droplet-based microfluidics in applied biological sciences (Table 9.6).

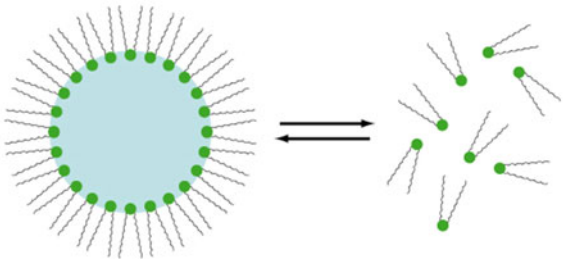

Fig. 9.8 Accumulation of surfactant molecules on the surface of aqueous droplets

Table 9.6 Publications on surfactant-stabilized, droplet-based microfluidics in the field of cell-biology and microbiology

Author	Assay	Year
Agresti et al. [33]	Directed evolution	2010
Kintses et al. [34]		2012
Fallah-Araghi et al. [92]		2012
Beer et al. [109] Kiss et al. [110]	Single-copy quantitative (RT)-PCR	2008
Brouzes et al. [46]	High-throughput single-cell cytotoxicity testing	2009
Mazutis et al. [76]	Isothermal DNA-amplification	2009
Tawfik and Griffiths [112]	In-vitro translation	1998
Dittrich et al. [113]		2005
Fallah-Araghi et al. [92]		2012
Mazutis et al. [76]		2009
Miller et al. [104]	High-resolution dose-response screening	2012
Pekin et al. [77]	Quantitative detection of rare mutants	2011
Granieri et al. [114]	Phage-display	2010
El Debs et al. [115]	Monoclonal antibody production and target binding studies	2012

All of the presented experimental setups rely on the same microfluidic unit operations, which are discussed below.

An important prerequisite for unit operations in emulsion-based microfluidics is a suitable carrier fluid/surfactant combination. Different combinations are employed, depending on the type of application—but only a few can be considered as prevalent: Mineral oil or hexadecane with Span80 as surfactant provide a simple and easily obtainable carrier fluid system, which can be applied in most assays, particularly those that do not include long droplet incubation periods [48, 61–65]. If higher droplet integrity is required, perfluorinated carrier fluids as FC-40, FC-77 or HFE-7500 (3M) are employed [66]. These oils exhibit very high chemical inertia and minimal water solubility, hence are less prone to droplet cross-talk. A further feature of perfluorinated carrier oils is their very high gas solubility, which can be of advantage if oxygen supply or gas formation of encapsulated microorganisms is critical. Biocompatible fluorosurfactants are often triblock copolymers composed of two perfluorinated polyether tails (PFPE) and a hydrophilic head, e.g. dimorpholino phosphate (DMP-PFPE) or polyethylene glycol (PEG-PFPE) [67–70]. They provide long-term droplet stability for many cell-based applications, but were until recently not commercially available, or only with rigorous restrictions (EA-surfactant, RainDance). PicoSurf^{TM} by Dolomite (Royston, England) fills that gap now.

Droplet generation is accomplished in most cases at cross-junctions by "flow-focussing" or at T-junctions, where the incoming aqueous stream is pinched off by the carrier fluid into monodisperse droplets [15, 71–73]. Due to hydrophobic channel surface modification (e.g. Repelsilan, Ombrello) and the presence of surfactant in the continuous phase, wetting of the channel walls by the emerging droplets is avoided. Even in cases where the droplet occupies the majority of the channel's cross section

("slug flow"), a lubricating layer of carrier fluid is maintained between the droplet and the channel wall.

Addition of cells or reactants to existing droplets can be accomplished by either pairing and merging targeted droplets with droplets delivering the contents in demand [74–77] or by dispensing the reagent or cell-containing fluid into targeted droplets passing a T-junction ("picoinjection") [78]. Independent of the merging technique applied, the targeted droplets have to be spaced with carrier fluid after reinjection into the chip, which can also be achieved at cross- or T-junctions, possibly arranged in a sharp angle to reduce shear stress. Mazutis et al. [76] used FC-40 perfluorinated oil with reduced surfactant concentration (0.27–0.55 % w/w EA-surfactant, Raindance) for droplet spacing, which leads to depletion of surfactant molecules on the droplet's surface upon reinjection. Droplets being generated on-chip using the same surfactant concentration are paired with the reinjected ones by flow-rate synchronization and droplet pairs are finally merged in zig-zag-shaped channels inducing local surfactant depletion and thus facilitating fusion of droplet interfaces. To diminish subsequent undesired merging of droplets in the collection chamber, carrier oil with high surfactant concentration (2.8 % w/w) was injected upstream of the zig-zag-structure. Since diffusion of the surfactant micelles to the droplet surface is a function of surfactant concentration and time [32], desired droplet coalescence in the zig-zag-channels is merely affected. The one-to-one droplet fusion efficiency achieved with this technique is ~99 %. To avoid such complicated and cumbersome flow arrangements, droplet merging or addition of fluids to existing droplets can be induced with electric fields generated by on-chip electrodes located near the point of droplet pairing or fluid dispensing, respectively [79–81]. By applying a pulsed electric potential of less than 3 V, Priest et al. [82] demonstrated targeted destabilization of inter-droplet lamella resulting in pairwise droplet merging in a hexadecane/saline system with 2 % Span80 as emulgator. In the same work it was shown that a 10 μm insulating layer between the electrodes and the channel does not impede electrocoalescence, since capacitive coupling carries the voltage to the rupture area when AC electric fields are applied. This way, undesired electrochemical side effects detrimental to living cells are also circumvented. In a series of experiments published by Ahn et al. [83], two droplet populations with different average size (50 and 25 μm) were generated on-chip and merged pairwise. Interestingly, the authors exploited the rheologic effect of smaller droplets catching up to larger ones, to reliably reduce inter-droplet distance and facilitate electrocoalescence of the two droplet species. Using hexadecane as continuous phase with 5 % (w/w) Span80 as surfactant, 100 V AC at a frequency of 100 kHz was required for successful merging at a rate of 100 droplet pairs/s. Given an electrode distance of 200 μm, the applied voltage accounts to a field strength of 500 kV/m. This concept is also applicable for reinjected droplets which were incubated off-chip to replace one or both of the on-chip generated droplet species. However, the discussed methods of droplet pairing and merging require precise fine tuning of flow rates for synchronization of the two droplet species, which is in every case prone to errors and occasionally yields two-to-one fusion events or unfused droplets.

In contrast, “picoinjection” provides an approach where only one population of droplets must be handled, since fluids containing cells or agents are directly dispensed into the reinjected droplets from a perpendicular and pressurized channel at a T-junction with adjacent electrodes generating an electric field [78]. As an additional benefit of this technique, injection can be performed at kHz-frequencies and in a selective manner, e.g. triggered upon detection of fluorescence. Moreover, serial and combinatorial injections are feasible by implementing additional picoinjection structures along the droplet-conducting channel. However, the authors do not describe how the pressure in the feeding channel is controlled, which is relevant for such complex dispensing patterns. The downside of this and similar techniques is the risk of cross-contaminations and carry-over-effects through the dispensing channel, but both can be considered as not important in case of the aimed screening for antibiotics: cross-contamination of filamentously growing, monolithic micropellets of Actinomycetes is unlikely and carry-over of potentially produced antibiotics would be subject to strong dilution and thus negligible. For completeness it should be mentioned that Sivasamy et al. [64] developed a passive merging structure for dosage of reagents that represents an intermediate between a droplet merger and a dispensing unit: aqueous droplets evolve from a feeding channel directly into a square-shaped chamber in downstream proximity, forming an uncurved interface with the carrier fluid (mineral oil + 2 % Span80), which exhibits a relatively low Laplace pressure jump. According to the authors, the interface can thus be easily disturbed by approaching droplets, resulting in a high probability of merging. However, we were not able to reproduce these findings when testing similar structures with FC40+PEG-PFPE surfactant as continuous phase.

Apart from incubation and detection—which is not subjected in this section—droplet sorting is the last required unit operation for a complete, surfactant-stabilized, microfluidic screening for novel antibiotic substances. Applied structures generally consist of a bifurcated microfluidic channel and adjacent eletrodes generating a pulsed, non-uniform electric field upon triggering [84–86]. Without pulse, droplets flow into the output channel with lower fluidic resistance, which is determined by downstream width and length of the channels. When the electrodes are energized, the respective droplet is polarized and dragged towards higher field strength, which is located near the output channel with higher fluidic resistance. By this, droplets of interest—e.g. selected on the basis of fluorescence detection—can be separated from the droplet population. The required voltages depend on the droplet velocity and volume, the viscosity of the continuous phase, the shape and position of the electrodes and the difference in fluidic resistance of the output channels. For sufficient displacement of 25 μm-diameter aqueous droplets in hexadecane, Ahn et al. [84] applied a 500 μs-pulse of 10 kHz and 700 V across the electrodes. This allowed for reliable droplet sorting at frequencies of 1,600 Hz.

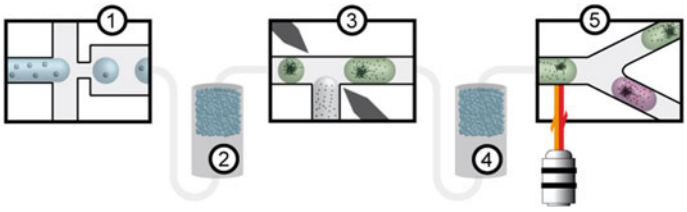

Fig. 9.9 Screening for antibiotic activity from soil-derived actinobacteria spores singularized and germinated in pL-dropltes: (*1*) droplet generation (*2*) incubation of spores (*3*) addition of reporter cells aided by electrodes (*4*) incubation of reporter cells (*5*) fluorescence detection and droplet sorting

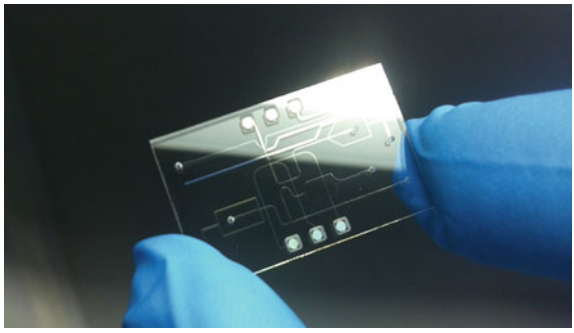

Fig. 9.10 Microfluidic fused silica chip

9.5.2 Screening for Novel Antibiotics with an Emulsion-Based Microfludic Approach

The general workflow for the emulsion-based search for novel antibiotics on the basis of the unit operations discussed above is depicted in Fig. 9.9. In contrast to screening in a micro-segmented flow, droplets are incubated in bulk and addition of reporter strains is assisted by electric fields.

To test the applicability of the surfactant-stabilized microfluidic concept for antibiotic screening, chips were designed for droplet generation, addition of reagents (dosage) and droplet sorting, according to the references discussed above (Fig. 9.10). Chips were fabricated by LioniX BV (Enschede, Netherlands) from fused silica wafers: the top-plate (0.5 mm) bears 50 μm deep channels generated by deep reactive ion etching using a 3 μm silicon layer as masking material. Vias for fluidic and electric contacting were created by powderblasting. Metallic electrodes were applied to the bottom-plate (1.0 mm) using a lift-off process on a sputtered stack of platinum (180 nm) on top of a titanium adhesion layer (15 nm). Prior to bonding of both halves, a layer of silicon oxynitride was deposited for levelling and insulation, using plasma-enhanced chemical vapor deposition. Before use, chips were rendered hydrophobic by rinsing with dimethyldichlorosilane (Repelsilan, Amersham Biosciences). Additionally, different devices were tested for droplet incubation: besides

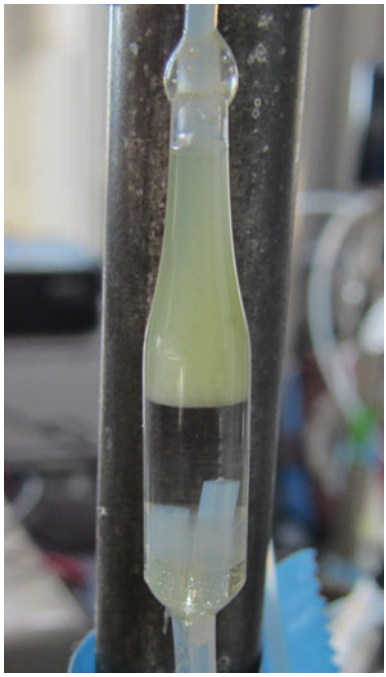

Fig. 9.11 Incubation vial made from a clear-glass micro-insert for HPLC-vials. Creamed droplets can be observed in the upper part

simply guiding droplets into PTFE-tubing (0.5 mm ID), droplets were transfered into 1.5 ml microreaction tubes or silanized pasteur pipettes followed by overlaying with pure and sterile culture medium to minimize droplet evaporation. For another home-made incubation device (in the following referred to as "incubation vial"), the tip of a clear-glass micro-insert for HPLC-vials (15 mm, 0.1 ml, VWR) was removed with a glass cutter and connected to PTFE-tubings after sealing the bottom with a silicone plug and epoxy resin adhesive (Fig. 9.11). Subsequently, the device was rendered water-repellent with Ombrello (moton auto-motive, Germany). Within this setup, no overlaying with aqueous medium was required.

The aforementioned microfluidic unit operations were evaluated with regard to their suitability for antibiotic screening from actinobacteria spores. Emphasis was given to droplet generation, incubation and reinjection, since these steps are prone to various stresses of physical and biological nature (Table 9.7) that influence droplet populations to an unknown extent. Hence, conditions for all subsequent operations are set during these initial steps, particularly by the degree of monodispersity of the resulting droplet population: high polydispersity will hamper reliable addition of reporter cells and is disadvantageous to the final step of droplet sorting. To assess the impact of spores and their germination on the monodispersity of a droplet population, we tested droplet generation from spore suspensions and subsequent incubation under diverse conditions.

Table 9.7 Physical and biological factors influencing droplet populations

Physical	Evaporation
	Satellite formation
	Ostwald ripening
	Merging (local surfactant depletion)
	Cross-contamination (through wetting)
	Cross-contamination (through dispensing channel)
	Diffusion, cross-talk
Biological	Gas formation due to metabolic activity
	Shrinkage due to metabolic activity
	Merging (increased surface tension through catabolites)
	Outgrowth of hyphae

Table 9.8 Composition of a model actinomycetes spore suspension used for microfluidic experiments

Strain	**Taxon**	**Produced antibiotics**
IMET40285	*Streptomyces gelaticus*	Elaiomycin
IMET40235	*Streptomyces griseus*	Cycloheximid
HKI0323	*Amycolatopsis lactamdurans*	Cephamycin C
HKI0040	*Streptomyces canus*	Amphomycin
HKI0016	*Streptomyces hygroscopicus*	Staurosporin
JA02640	*Streptomyces californicus*	Viomycin
IMET40177	*Streptomyces griseorubiginosus*	Cinerubin
HKI0216	*Kitasatospora azatica*	Alazopeptin
HKI0423	*Nonomuraea recticatena*	
HKI0269	*Streptomyces celluloflavus*	Aureothricin
IMET41584	*Streptomyces albus*	Salinomycin

Without applying spores, it was found that a flow rate of 195 nl/s for the carrier oil (HFE 7500 + 1 % PicoSurf) and 94 nl/s for the aqueous phase (MMM) gives the best results with regard to polydispersity. Droplets were produced at frequencies above 500 Hz, exhibiting a polydispersity of 1.15 %, which is clearly below the accepted standard of 2–3 % according to literature [87]. When a model spore suspension (in MMM) (Table 9.8) was employed instead of water as aqueous phase, similar values for the polydispersity were achieved, indicating that the presence of spores does not affect droplet generation. However, larger droplets bearing an obtrusive piece of mycelium were observed with non-significant frequency. These irregularities are likely to be minimized with more rigid centrifugation and filtration during spore extraction, which reduces the proportion of loose filaments in the suspension. Although low polydispersity values were obtained within each experiment, we observed high fluctuations in average droplet size between different droplet generation experiments: at various combinations of aqueous phase (pure MMM, MMM with spores and MMM with reporter cells), incubation device (Pasteur

Table 9.9 Restraints of droplet-based microfluidics with regard to cell-based assays

limited mass transfer
no agitation
limited sensing (pH, pO_2, etc.)
pH-shift
depletion of growth media
biofouling of surfaces
clogging

pipette, microreaction-tube and incubation vial) and surfactant concentration (1 and 1.25 %), the apparent mean droplet size varied between ~115 and ~139 pL, although the same flow rates were applied. Even with identical setups, the measured average droplet sizes revealed recognizable inter-experimental variations. While these can only derive from image analysis-related issues (variations in light intensity, focus plane, detection algorithm), greater variations at unequal experimental setups could also be attributed to changes in backpressure (due to varying incubation device and length of output tubing) and viscosity of the aqueous phase. The variations in the mean volume of different droplet populations complicate comparing inter-experimental changes in polydispersity, but they do not impede intra-experimental comparisons of a single droplet population after generation and incubation.

To evaluate the physical impact of droplet incubation and reinjection on the polydispersity of a population, droplets generated from pure MMM were incubated for 0, 24 and 48 h before partial reinjection (Fig. 9.12). To our surprise, droplets that were immediately (0 h) reinjected showed the highest divergence in average volume (110.8 pL) compared to the measurement upon droplet generation (115.61 pL). With increasing incubation time, the mean droplet volume approached the original value. These findings cannot be ascribed to a certain physical effect rather than variations in imaging conditions. More importantly, polydisperity did not increase substantially with prolonged incubation time: upon droplet generation, a value of 1.24 % was measured, while after 48 h the polydispersity was 1.30 %.

These results indicate that Ostwald ripening occurred only to a negiglible extent within this incubation period. Nevertheless a marginally increased number of satellite droplets was observed, which probably emerge due to shear upon reinjection and slight over-concentration of surfactant in the carrier oil. Additionally, a small number of larger droplets was detected during each reinjection round. Since their volume was not a harmonic multiple of the average droplet size, they did not originate from droplet merging events. Their occurrence remains to be elucidated, although they did not impede further unit operations. In the next step, the impact of actinomycetes on the droplet population was investigated: instead of using pure medium as aqueous phase, droplets were generated from a spore suspension (in MMM). Subsequently, the droplets were transferred to an incubation vial allowing for spore germination (at room temperature). As expected, droplets are nearly as monodisperse as upon generation when reinjected immediately, since germination has not occured yet.

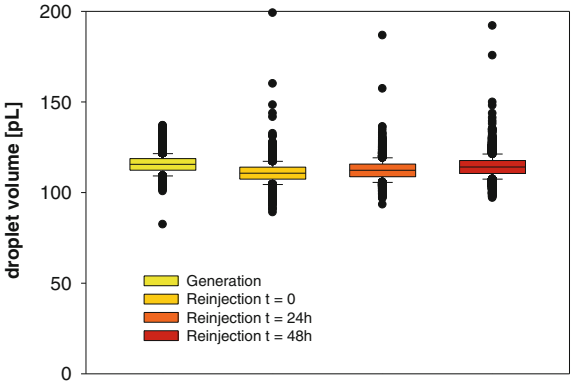

Fig. 9.12 Droplet volumes upon generation and after reinjection. Aqueous phase: pure MMM

Even after 24 h of incubation the polydispersity increased only from 1.12 % (upon generation) to 2.07 %, and first droplets bearing mycelium were observed (Fig. 9.13). After 48 h incubation, the majority of droplets showed growing actinomycetes. Interestingly, droplets with microcolonies shrank while empty droplets experienced an increase in volume (Figs. 9.14 + 9.15). This phenomenon, which is numerically reflected in an elevated polydispersity of 9.27 %, can be ascribed to the metabolic activity of growing bacteria: carbon sources are partly catabolized to highly diffusive products (mainly CO_2) that easily escape the droplet. As a result, the osmolarity decreases, leading to an efflux of water towards unoccupied droplets, since they exhibit a relatively higher osmolarity [88, 89]. In extreme cases, the mycelium occupies the whole volume of a droplet, which is thus deformed in its surface. However, undesired outgrowth of hyphae is only observed in very rare cases after extensive incubation periods. It should be pointed out that hardly any fused droplets were observed, indicating that the employed model actinomycetes did not produce any metabolites that significantly affect droplet integrity. Whether this finding also applies to larger model libraries or even to the immense diversity of actinomycetes found in soil samples remains to be investigated, but it provides first evidence that maintaining microcultures of unknown actinobacteria in surfactant-stabilized droplets is feasible.

A potential caveat to both micro-segmented flow and the emulsion-based approach is also related to incubation: secondary metabolites with antimicrobial activity emerging from the droplet of origin to other droplets in close proximity might cause false negative or positive hits, depending on the antimicrobial potency as well as the mobility of the respective molecule. This phenomenon, also termed "cross-talk", was recently investigated by various researchers. Bai et al. [66] tested the retention of fluorescein in trapped droplets dispersed either in mineral oil/Span80 or FC77/EA-surfactant, respectively. As already indicated above, the author found that the perfluorinated carrier oil (FC77) exhibited much lower transport of fluorescein molecules, due to lower fluidity at the water/oil interface. Nevertheless, Courtois et al. [90] and Skhiri et al. [91] found that surfactant concentrations widely exceeding the critical micellar contration (CMC) can enhance cross-talk by formation of

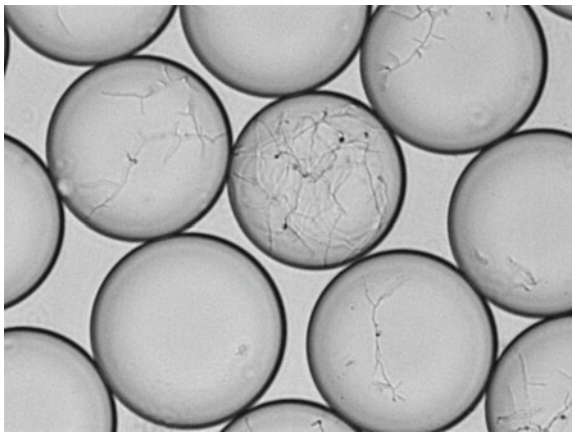

Fig. 9.13 MMM-droplets with mycelium or micropellets (after 24 h)

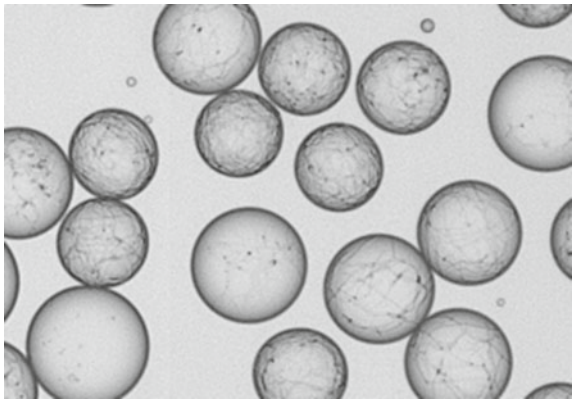

Fig. 9.14 MMM-droplets with mycelium or micropellets (after 48 h)

reverse micelles entrapping and transporting molecules that are dissolved in the droplets. Together with diffusion, this mechanism is claimed to play the key role in inter-droplet exchange of molecules. Both authors also present a counteracting measure to molecule transport between droplets: by addition of BSA to the aqueous phase, the retention of resorufin was enhanced 18-fold [91], and transport of fluorescein could also be reduced signifcantly. Since this finding is ascribed to the general property of BSA to increase solublity of other molecules, it can be assumed that this also applies for unknown antimicrobial substances entrapped in droplets. Although molecule mobility is related to its charge and is thus unpredictable for the bulk of unknown molecules produced by actinobacteria, cross-talk can probably be reduced to acceptable dimensions by employment of BSA or similar additives.

In a further step, addition of reporter cells to droplets containing germinated spores with a picoinjector was tested. As mentioned above, high polydispersity of the incoming droplet population was assumed to be detrimental to reliable picoinjection. Different droplet sizes lead to varying inter-droplet distances after spacing which

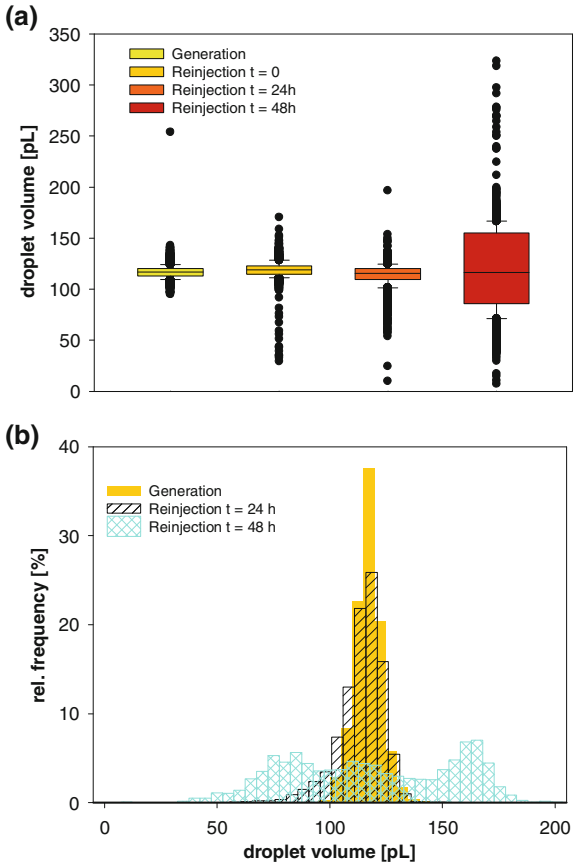

Fig. 9.15 Droplet volumes upon generation and after reinjection (0, 24 and 48 h). Aqueous phase: MMM + spores. B: Distribution of droplet volumes upon generation and after incubation

in turn results in the addition of unequal fluid volumes. If the diameter is smaller than the channel cross-section, the droplet might even not come into contact with the meniscus of the dispensing channel and thus not be subjected to reporter cell addition. However, upon reinjecting 3-day old droplets generated from the model spore suspension with 140 picolitre average volume, all droplets were still large enough to occupy the entire cross-section of the incoming channel upstream of the picoinjection structure (size 50 μm). The outgoing, plug-shaped droplets appeared equal in volume. Yet, addition of variable amounts to the droplets would not be detrimental to the overall assay performance, since cell densities will equalize with growth time in droplets without antibiotic activity, diminishing the risk of false positives. However, in case droplet polydispersity becomes an obstacle in future assays—e.g. by limiting throughput—droplets can be sorted according to their size prior to each reinjection round as counteracting measure. The functionality of passive, size-dependent sorting structures was demonstrated recently [92–94].

The last remaining unit operation to be tested is sorting of droplets representing a hit. Again, it must be assumed that high polydispersity of incoming droplets is challenging with regard to droplet sorting. However, as in the case of reporter cell

picoinjection, passive droplet-sorting according to size prior to fluorescence guided sorting might be helpful. The maximum frequency of reliable droplet separation from the main population has to be assessed and is surely dependent on the exact shape of applied channel and electrode structures as well as the droplet volume and velocity. Difficulties might arise from the contradiction of reporting principle and detection mechanism: since reporter cells are inhibited, droplets containing an antibiotic will be non-fluorescent, while the evaluation of droplets is fluorescence-based.

However, this issue might be easily solved by detection and sorting of the fluorescent droplet species, which also leads to pooling of non-fluorescent droplets at the other end. Nevertheless, this approach would result in an extremely high frequency of sorting events, which inflicts higher demands on the sorting periphery. The feasibility of such an ultra-high-frequency sorting must be tested. Alternative ways to tackle this problem might include general addition of a fluorophore that emits at a different wavelength or detection of all droplets by capacitive sensing prior to fluorescence analysis [63].

Once droplets of interest are sorted into the "value" outlet, they have to be separately extractable so that they can serve as inoculum in an upscaling chain—starting from a well of a microtiter plate, for example. A suitable chip-to-world interface remains to be developed, which is also a requirement for other droplet-based microfluidic assays and thus only a matter of time.

9.6 Summary and Outlook on Antimicrobial Screenings in Micro-Segmented Flow and Emulsion-Based Systems

The constant emergence of new, life-threatening pathogens and resistance mechanisms requires the development of novel antibiotic substances and substance classes. Target-oriented approaches, as they were postulated at the beginning of the 1980s, failed to reveal new antimicrobials. Despite the discovery of suitable candidates for target-inhibition, most substances were not able to penetrate the bacterial cell wall. Hence, leading experts proposed the return to whole-cell-based screening of soil-derived actinobacteria—an approach that delivered the majority of all discovered antibiotics so far. However, classic screening of actinomycetes is cumbersome and suffers from low throughput, which limits its success rate. By providing millions of microscaled reaction compartments, droplet-based microfluidics allows for high-throughput cultivation of Actinobateria and promises subsequent whole-cell testing for production of antimicrobial substances. Here, micro-segmented flow, implemented on a monolithic chip device, and an emulsion-based approach were tested for antibiotic screening capabilities by separately investigating all contributing unit operations.

Droplet generation and incubation led in both discussed systems to germination of encapsulated spores and formation of micropellets. Penetration of the droplet/carrier oil interphase by growing hyphae was only observed in rare cases after very long

incubation periods of several weeks. However, subsequent addition of reporter cells could not be achieved with micro-segmented flow, since inter-droplet distances were not uniform and undesired droplet fusions occurred. Several methods to resolve this conflict were proposed, including the introduction of a third immiscible phase to separate droplets and external incubation in PTFE tubing. To test the system for general detectability of antibiotic activity, droplet series with a concentration gradient of the protein-synthesis-inhibitor nourseothricin and culture supernatant of nourseothricin-producing *S. noursei*, respectively, were generated. After addition of *E. coli* reporter cells and subsequent incubation, droplets were analyzed for fluorescence. For both, the pure nourseothricin and the culture supernatant, the minimal inhibitory concentration could be clearly determined.

For the modular, surfactant-stabilized system it was shown that growth of actinomycetes leads to an increase in droplet polydispersity. Nevertheless, by applying moderate incubation times, the polydispersity can be kept in an acceptable range, still allowing for reliable addition of reporter cells by picoinjection. In case droplets with higher polydispersity must be handled—due to prolonged incubation times for example—antecedent size-dependant droplet-sorting with passive sorting structures is proposed. Fluorescence-dependent sorting of droplets with pulsed electric fields was already demonstrated in several studies, but applicability for polydisperse droplet populations bearing mycelia of actinomycetes still has to be confirmed. To get hold of droplets sorted on-chip, an interface allowing for selective guidance of single droplets, e.g. in the wells of a microtiter plate, needs to be developed.

Issues and questions concerning the magnitude of undesired droplet-cross-talk, quality of the read-out provided by different reporting principles and achievable throughput need to be resolved. Moreover, accessible diversity of actinobacteria and frequency of unknown species found in droplets has to be investigated, e.g. by pyrosequencing of 16S-rRNA fragments. If required, pooling of soil samples, variation of media composition and employment of streptomycete-specific phages [95] might further increase the probability of discovering yet unknown species. Expression of orphan pathways might be stimulated through addition of aqueous soil extracts [96, 111]. As a promising variation of the presented screening approach from actinobacteria spores, high-throughput-investigation of soil-derived metagenomes in prokaryotic hosts might be taken into consideration [97, 98], although probabilities of generating hits must be thoroughly evaluated [99].

The general feasibility of a droplet-based microfluidic screening of soil-derived actinobacteria spores for novel antimicrobial substances was confirmed. Although further development and fine-tuning is required, the presented case-studies raise the prospect of a highly efficient assaying system as a source of new active substances against emerging pathogens.

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