A chemical waveform synthesizer

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Algorithms and methods were developed to synthesize complex chemical waveforms in open volumes by using a scanning-probe microfluidic platform. Time-dependent variations and oscillations of one or several chemical species around the scanning probe, such as formation of sine waves, damped oscillations, and generation of more complex patterns, are demonstrated. Furthermore, we show that intricate bursting and chaotic calcium oscillations found in biological microdomains can be reproduced and that a biological cell can be used as a probe to study receptor functionalities as a function of exposure to time-dependent variations of receptor activators and inhibitors. Thus, the method allows for studies of biologically important oscillatory reactions. More generally, the system allows for detailed studies of complex time-varying chemical and physical phenomena in solution or at solution/surface interfaces.

Oscillatory reactions, in which the concentrations of chemical species vary nonlinearly with time, are plentiful. Some are found in pure chemical systems such as the famous autocatalytic Belousov–Zhabotinskii reaction, but many more are found in biology (1). Biological processes that exhibit oscillatory behavior exist in all living species, from mammals to bacteria (2). Examples include circadian rhythms (3), oscillations of cAMP (4) or Ca2+ (5), and the pulsatile nature of hormone secretion (6). Lately, several intracellular signaling pathways have been characterized and found to have oscillatory behavior (4, 7–10), and the importance of the dynamic behavior of pathway systems has become increasingly clear (11). However, despite the importance of time-dependent variations of chemical substances in biological systems, there are no methods that directly can generate and emulate such complex variations in the concentration of key species on a relevant time (i.e., milliseconds to minutes) and length (submicrometer-to-micrometer-sized objects such as single organelles and cells) scale for experiments on, e.g., receptor functionality and pattern decoding (12), oscillatory, pulsatile, and chaotic Ca2+ fluctuations (5), disruption of secretion patterns in disease states; for elucidating input and output relations in system biology (11–13); and for precisely mimicking release of signaling molecules (14). With current methods for solution exchange around single cells or cell fragments such as photolysis (15, 16), U-tubes (17), puffer pipettes (18), multibarrels (19), or liquid “filament switches” (20), it is difficult to achieve a complex, rapid, and well-controlled waveform exposure due to a too small number of solution environments, inability to switch from high to low concentrations, lack of precision, or poor time resolution. Furthermore, the generation of overlapping concentration patterns of multiple species as a function of time, which is an important feature in biological applications where different substances often interact in a concerted manner, is very difficult with these methods.

Here, we show how a microfluidic platform, previously described in refs. 21 and 22, can be used as a chemical waveform synthesizer. The platform comprises a microfluidic chip that creates chemical landscapes in open volumes and a computer-controlled scanning stage for translating a probe/object within these landscapes. The landscapes are created through coalescence of multiple laminar streams of different constituents as they exit from a channel array on the chip. The concept of joining multiple flows to generate chemical gradients has been used previously in closed channels (23–25) and applied to label different subcompartments of a single cell (26) and to study chemotaxis (27) and some physical properties of solutes (25). Furthermore, chemical gradients have been created in closed microfluidic channels by isoelectric focusing (28, 29). With the synthesizer presented here, different probes such as cells or cell fragments (or any other physical probe of nanometer-to-micrometer dimension) can be exposed to complex chemical waves having single or multiple constituents. These waves can be designed to precisely mimic in vivo conditions, such as release and uptake of signaling molecules and oscillations in the concentrations of biologically active molecules to create complex man-made stimulation patterns to perform the above-suggested experiments and studies. In a more general way, the system can be applied to any problem of time-dependent changes of a chemical species in solution phase or at solution/solid-phase boundaries. Also, time-varying patterns proposed in mathematical models of biological systems can be emulated and validated by using this system.

Materials and Methods

Experimental Set-Up. A microfluidic chip was mounted on an inverted microscope stage (Model DM IRB, Leica, Wetzlar, Germany) equipped with a motorized scanning stage (Scan IM 120 × 100, Marzhauer Wetzlar, Wetzlar-Steindorf, Germany). A detailed description of the chip and the scanning stage, the properties of the generated laminar flow, operational guidelines, and limitations for the chip can be found in ref. 21 and in the legend of Fig. 4, which is published as supporting information on the PNAS web site. The chip contains an array of microchannels. Each channel connects a sample reservoir with an open volume. The number of channels as well as their dimensions can be varied to fit a specific experiment. The present work is based on a device having 16 channels with a center-to-center distance of 72 μm and a height of 60 μm. To use the chip, the sample reservoirs and the open volume are filled with the desired solutions, and a flow through the channels, from the sample reservoirs to the open volume, is started by applying pressure to all of the sample reservoirs simultaneously using a diaphragm pump (Cellelectron, Gothenburg, Sweden). Presupposing that the chip is run in the low-Reynolds-number laminar flow regime (see Fig. 4 for details), the fluids exiting the channels into the open volume couple to create one laminar patterned stream. Scanning of probes between different solution environments in the patterned stream was accomplished by keeping the probe fixed while moving the scanning stage with the microfluidic chip placed on it. The movement was controlled by a computer using the software HYPERTERMINAL (Microsoft). An EPC10 triple patch-clamp amplifier (HEKA Elektronics, Lambrecht/Pfalz, Germany) was used for the amperometric concentration measurements, and an Axopatch 200 A patch-clamp amplifier (Axon

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Instruments, Union City, CA) was used for the patch-clamp measurements.

Electrochemical Measurements. A buffer containing 25 mM KH$_2$PO$_4$, 25 mM K$_2$HPO$_4$, and 0.1 M KCl (pH 7.2) was used with varying concentrations of L-dopamine (Fig. 2) or hexamminerruthenium(III)chloride (Fig. 3, 4). Carbon fiber electrodes (ProCFE, Dagan Corp., Minneapolis) having a cylindrical electro-active area (5-µm diameter, 30-µm length) were positioned outside the channel outlets by using micromanipulators. During measurements, a potential of 0.8 V (L-dopamine) or –0.3 V [hexamminerruthenium(III)chloride] was applied to the electrode (vs. Ag/AgCl). The sampling frequency was 200 Hz, and all curves were low-pass filtered at 100 Hz for analysis and presentation. In the curves presented in Fig. 2A and D, each period was individually normalized to account for noticeable electrofoiling of the electrode during these experiments. All measurements were performed in a plane 30 µm above the bottom of the chip. In-channel flow velocities ranged from 260 µm/s to 18 mm/s, and the maximum scan speed used was 1 mm/s.

Patch Clamp Experiments. Adherent WSS-1 cells with an average diameter of ~10 µm were cultivated according to standard procedures. Before the patch-clamp experiments, cells were washed and detached in a Hapes saline buffer containing 10 mM Hapes, 140 mM NaCl, 5 mM KCl, 1 CaCl$_2$, 1 mM MgCl$_2$, and 10 mM D-glucose (pH 7.4). The diameters and the resistances of the patch-clamp pipettes were ~1 µm and ~4 MΩ, respectively. The patch-clamp electrode solution contained 100 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 11 mM EGTA, and 10 mM Hapes; pH was adjusted to 7.2 with KOH. All experiments were performed at room temperature (18–22°C). Signals were recorded at a holding potential of ~40 mV and were digitized and stored on the computer hard drive (sample frequency 5 kHz, filter frequency 1 kHz using a four-pole Bessel filter). Data analysis was performed by using CLAMPFIT 8.1 software (Axon Instruments).

Results and Discussion

Creation of Spatial Concentration Landscapes. When operated in the regime of laminar flow, the fluids originating from different sample reservoirs couple to create one patterned laminar stream when exiting the channels. In the open volume, the stream advances in both the forward and the upward direction (the y and z directions, respectively) with a velocity profile described in ref. 21. The loading scheme of the reservoirs defines an input matrix, and the laminar flow, exiting from the channels and extending into the open volume, forms a 3D concentration space. Below, we discuss concentration variations in the output volume in terms of 2D concentration landscapes, being cross sections of the concentration space for different heights above the bottom surface of the open volume (fixed z-values). Although it should be remembered that the detailed appearance of these landscapes varies between different heights, the only practical implication is that the same z-value has to be used in an experiment to achieve reproducibility. In Fig. 1, a schematic of a loading pattern of a microfluidic chip, presented as an input matrix, and a resulting x-y-concentration landscape is shown (fixed z-value).

The number, type, and concentration of substances that can be loaded per sample reservoir in the microfluidic device are restricted only by solubility limits and possible interactions. By loading multiple substances in the sample reservoirs, overlapping concentration profiles can be created. The concentration c of a loading substance in the output landscape is a function of the input matrix INS (the indexes N and S refer to channel and substance number, respectively), the flow velocity $v_{flow}$, and the position/coordinate $r = [x, y]$, where x and y are the horizontal plane coordinates (see Fig. 1): $c = f(IS, v_{flow}, r)$. However, for a chosen input matrix and a fixed $v_{flow}$, we can write $c = f(r)$, where f depends on the input conditions. Close to the channel outlets there is a region where the concentration of the respective substance varies in discrete steps along the x-axis. Here, each column in the input matrix has been mapped to a finite element with a bit-size equaling the center-to-center distance (h) of the channels. As the fluid travels in the y direction, diffusion successively mixes the content of adjacent elements. For a given solute/solvent system, the flow velocity determines the degree of mixing at different y-coordinate values. Increasing/decreasing the flow velocity results in a stretching/compression of the y axis in Fig. 1.

Creation of Spatial Concentration Profiles. To predict the entire concentration landscape $c = f(r)$ for a specific set of input parameters, computer simulations must be used (21). However, when we are scanning the probe close to the channel outlets where negligible diffusion mixing has occurred, a desired concentration profile along the x axis, $c = f_{desired}(x)$ for $x = [a, b]$, can be obtained in the following way. First, the interval $x = [a, b]$ is partitioned into subintervals $I_i = [x_i - 1, x_i]$ of length $x_i - x_{i-1} = h$. The number of subintervals should equal the number of microchannels that are to be used for the design. Then, a piecewise constant approximation is made to $f_{desired}$ using this partition. The approximation can then be directly transferred to the input matrix, with each channel corresponding to a subinterval and the loading concentration for the respective channels being given by the coefficients in the mathematical approximation. In this way, concentration profiles reflecting any waveform can be created; the quality of the approximation is decided by the...
channel size and number of channels. In principle, each channel corresponds to a finite element, and the fluid exiting the channels together builds a profile in the way basis functions are used to build approximations in mathematics (30).

Creation of Concentration Waves in the Time Domain. The scanning stage has submicrometer precision and can move a probe in the x and y directions in the chemical landscape such that it meets a well-defined sequence of different solution environments. When the probe is moved between different positions, r becomes a function of time; \( r(t) = [x(t), y(t)] \). The probe then will experience a concentration wave in time; \( c(t) = f(r(t)) \), being a function of both the output landscape and the probe trajectory. For a given output landscape, which is determined by the input matrix and the flow velocity, how should then a probe trajectory be chosen such that the probe experiences a desired concentration wave and, vice versa, how should the concentration landscape vary for a given probe trajectory?

The answer can be found by taking the time derivative of c, to get the differential equation

\[
\frac{dc}{dt} = \frac{dc}{dr} \frac{dr}{dt}
\]

Given two of the factors \( dc/dt \) (how concentration varies in time around the probe), \( dc/dr \) (the gradient of the concentration), or \( dr/dt \) (the velocity of the probe), the differential equation gives the unknown factor that indicates either what concentration change will be experienced by the probe, how the concentration along the moving path should change, or how to choose a trajectory for the scanning probe.

Examples of Waveform Creation. To experimentally verify that complex chemical waveforms can be created, we loaded the sample reservoirs with an electroactive substance (L-dopamine, oxidization potential +0.8 V vs. Ag/AgCl, or Hexaammineruthenium(III)chloride, reduction potential −0.3 V vs. Ag/AgCl.) and scanned a 5-μm-diameter carbon fiber electrode outside the channel exits while detecting concentration levels amperometrically. Fig. 2A shows the creation of a profile including an approximation to a half period of a sine wave \( c = A\sin(2\pi t/T) + A \), where \( A \) is the amplitude, \( \lambda \) is the wavelength, and \(-\lambda/4 < x < \lambda/4 \) along the x axis by loading 16 channels with different concentrations of the electroactive species. The half period covers 13 channels (middle of channel 2 to middle of channel 15), and thus the sine wave has a wavelength of \( \lambda = 2 \times 13 \) h, which is 1,872 μm for the used chip. A perfect fit to the desired sine wave can be obtained by using moderate diffusion smearing through scanning at a distance further away from the microchannel exits or lowering the flow velocity (blue curve in Fig. 2A).

If a probe is scanned along the half sine wave profile with a constant velocity \( v \) giving a movement described by \( x(t) = vt \) (y is fixed), the profile will be mapped to the time domain so that \( c(t) = A\sin(2\pi t/T) + A - \lambda/4 < t < \lambda/4 \) (Fig. 2A). When scanning repeatedly, a full sine wave is obtained (Fig. 2A Right). The frequency of the wave is controlled by the scan speed. This example illustrates how patterns periodic in time can be created from nonperiodic landscapes by repeating the same trajectory several times. Scans can continue as long as the chemical landscape is stable, i.e., as long as the supply of fluid in the sample reservoirs lasts. Sample volume is a mere design issue, and for experiments consuming large volumes of sample solution, external sample reservoirs can be used.

Another example of a periodic wave created by building a half period in space and performing back and forth scans is the “devil wave” (Fig. 2B). Here the probe was moved close to the channel outlets where the concentration varies in steps with a bit-size of 72 μm (corresponds to \( h \) for the used device). The scan speed was 1 mm/s, giving a time resolution \( 1/h \) of 72 ms and a wave frequency \( f(=v/\lambda) \) of 0.46 Hz. Decreasing the bit-size \( h \) or increasing the scan velocity \( v \) would result in higher time resolution and higher frequency (21). The lower limit of \( h \) is set by manufacturing limits and is less than a few micrometers (31).

The upper limit on velocity is given by the point where solution exchange around the probe is incomplete as a result of problems with exchanging the unstirred layer, eddy formation behind the probe, or simply that the time it takes for new fluid to enclose the probe is too long compared with the residence time in the solution zones. Solution exchange depends on, among other factors, the size, and shape of the probe, diffusion coefficients, and channel flow velocity (32–34).

A limitation with using backward and forward scans along the x coordinate at constant velocity is that the achieved wave necessarily will have mirror symmetric periods as demonstrated above. To obtain nonsymmetric and more complex patterns, the scanning velocity can be exploited to control waveform shape. The most straightforward example of waveform shaping by velocity variations is the creation of different waveforms from a concentration ramp in space described by the function \( c = f(x) = kx \), where \( k \) is a constant defined by loading a sequence of channels with increasing concentrations (Fig. 2C Left). The linear relationship between concentration and position makes waveform design easy because concentration variations can be directly translated to movement in the x direction (\( x(t) = c(t)/k \)). Scanning across the ramp with constant velocity so that \( x(t) = vt \) results in a concentration ramp in time (\( c(t) = f(x(t)) = kvt \)). By repeating this movement back and forth, a “saw-toothed” wave in time is generated. Changing the scan speed results in a changed frequency whereas alteration of the scan length modulates the amplitude (Fig. 2C Right). Scanning across the same concentration ramp with an identical trajectory but with a velocity chosen such that the probe position in terms of time, \( x(t) \), is described by \( x(t) = A\sin(2\pi t/T) + A \), where \( T \) is a temporal period, \( A \) is a constant, and \( t = [a, b] \) instead generates a sine wave (\( c(t) = kA\sin(2\pi t/T) + kA \)) (Fig. 2D Right). The velocity giving the right moving pattern is obtained through differentiating \( x(t) \); \( v(t) = (dx/dt) = (2\pi/T) \times Acos(2\pi t/T) \). If \( x(t) \) is hard to describe mathematically, the derivative complex or functions cannot be given as input to the scanning stage; instead, a piecewise linear approximation to \( x(t) \) can be used to obtain a proper velocity scheme. Dividing the time interval \( t = [a, b] \) into an arbitrary number of subintervals \( I_i = (t_{i-1}, t_i) \) with arbitrary lengths \( h_i = t_i - t_{i-1} \) and making a piecewise linear approximation on this partition yields an approximate velocity for each subinterval, given by the slope of the respective approximation line, \( v_i = (x_i - x_{i-1})/(t_i - t_{i-1}) \), and a trajectory determined by the x coordinates at each subinterval boundary (\( x_{i-1} \) and \( x_i \)) (Fig. 2D Left).

Thus, the probe should be moved between position \( x_{i-1} \) and \( x_i \) with a velocity \( v_i \) which will take the time \( t_i - t_{i-1} \). This method has several advantages, such as ease of generation of waves with nonsymmetric periods, and it allows for an unlimited number of modifications of the waveform during a scan. However, because the method is based on shaping a waveform by probe movement, it is not optimal for creating independent simultaneous concentration changes of multiple species around probes.

So far, two approaches for simple, rationalized waveform design, both involving scanning in the x direction only, have been discussed. Several other approaches exist. For example, with control over the scanning trajectory, many different waveforms can be obtained from a simple alternated loading pattern, e.g., a periodic square wave (by scanning in the x direction close to the channel exits), damped oscillations (by cross scanning), a concentration ramp (by scanning in the y direction), and sine-like waves (by scanning along diffusion-smeared square-wave-profiles) (Fig. 2E). The waveforms obtained in these cases can be understood qualitatively with some basic knowledge in dif-
fusion theory and fluidics and can be decided quantitatively by computer simulations.

**Mimicking Time-Dependent Changes of Solutes in Biological Systems.** To study biological processes, it is useful to mimic physiological conditions. Although periodic chemical waves are most common, both bursting and chaotic oscillations have been observed (2). To demonstrate the functionality of the chemical waveform synthesizer, we chose to recreate bursting and chaotic calcium ion oscillations from a model by Houart *et al.* (35). The calcium ion oscillations were mimicked by using the scanning velocity to control waveform shape from an input matrix defining a broken...
concentration ramp (Fig. 3A Upper). A concentration ramp having two different slopes was used to cover the dynamic range in concentration change in the reproduced waveform. Switching between different waveforms and periodicities, mimicking bifurcations or phase transitions, can easily be accomplished within one scan. Such switching was illustrated by combining the bursting and chaotic waves proposed by the model into one scan (Fig. 3A Lower).

To further demonstrate the functionality of the chemical waveform synthesizer in biological studies, we performed whole-cell patch-clamp experiments. Ligand-gated ion channel receptors are suitable targets for synthetically generated waveforms because they show immense complexity in their gating kinetics depending both on concentration and exposure time. We have shown previously that the combination of microfluidics and patch clamp can increase the data throughput and experimental control (22). In this experiment, WSS-1 cells expressing the GABA<sub>A</sub> receptor were exposed to overlapping concentrations of an agonist (β-alanine) and a competitive antagonist (bicuculline) (Fig. 3B). As can be seen from the current trace, β-alanine opens the ion channel while increasing concentrations of bicuculline inhibit the β-alanine response. Thus, the waveform synthesizer allows for detailed studies of the gating properties of the receptor where the concentration of each species can be spatially and temporally controlled with high resolution. For example, it can be used to exactly mimic the way receptors are exposed to stimulus in vivo and to study how they respond to single or multiple drugs applied in a complex fashion.

When using biological probes, the stability of the probe itself might be what limits scan speed and flow velocity. In the specific case of whole-cell patch-clamp recordings, a study of the influence of laminar flow in a microfluidic device identical to the one used in the present study showed that flow velocities up to at least 10 mm/s can be used (36). However, it should be mentioned that much higher flow velocities are routinely used for solution exchange around biological probes when using older solution exchange techniques (17).

**Conclusions**

In conclusion, we have shown how chemical waves can be synthesized either in space as a profile by choosing a proper input matrix or in time by control of both output landscape and scanning trajectory. The platform allows for studies of complex time-varying chemical and physical phenomena in solution or at solution/surface interfaces. For example, in vivo waveforms can be mimicked and presented to biological probes in vitro to study effects of different time-varying patterns. In the perspective of systems biology, the synthesizer can be used to elucidate, e.g., robustness and the relation between input and output parameters. By using overlapping concentration pro-
files, probes can be exposed to a multitude of independent signals simultaneously, which allows for design of versatile and flexible experimental systems in studies of time-varying chemical patterns. The present chemical waveform synthesizer comprises a chip with a 1D channel array. By changing to 2D, the flexibility in waveform creation would further increase due to an increase of the number of neighboring channels to which the probe can be moved. Thus, repeated scans between mirror symmetric periods of different species around probes.

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