9. Appendix

9.1 Laboratory-internal operating procedures

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9.1.1 LOP of building adsorbing heads for the SPATS

"Protocol for the fabrication of adsorbing head devices for the SPATS"

A. Material

- Universal compressed gas can (210g/400ml) + pistol adaptor for compressed gas can (Propan/Butan gas can with thread, Art. Nr. 52109, CFH Löt- und Gasgeräte GmbH, Offenau, Germany)
- Glass capillary tube (Kapillaren zur Schmelzpunktbestimmung, open at both sides, AD 1,75 LG 100mm, 1000 pieces, Hirschmann Laborgeräte GmbH & Co.KG, Eberstadt, Germany)
- Metal block device with fixed conventional micrometer step motor for high-precision XY-adjustment; metal block for bending of glass capillary tubes
- Sharp/spiky item (e.g. needle or other) used for applying the adhesive
- Glass Petri dish used as platform for applying the adhesive
- Scalpel/tiny slotted screwdriver for adjusting screws of devices (screw threads)
- Copper hole-rings (AGAR scientific Ltd., Stansted, Essex, U.K.; ordered from PLANO GmbH, Wetzlar, Germany)

  G2605C 1500µm hole copper 3.05mm
  G2600C 1000µm hole copper 3.05mm
  G2680C 800µm hole copper 3.05mm
  G2660C 600µm hole copper 3.05mm
  G2630C 300µm hole copper 3.05mm
- Copper meshes (G2786C 2000 square mesh copper 3.05mm, AGAR scientific Ltd., Stansted, Essex, U.K.; ordered from PLANO GmbH, Wetzlar, Germany)
- UV lamp 220V 50Hz, 230V 60Hz (System Papst-Motor Typ 8550, Papst-Motoren GmbH & Co.KG, St. Georgen, Germany)
- Adhesive = Norland optical adhesive (ultraviolet curing) 88, LOT 164 (Norland products Inc., Cranbury, NJ, USA)

  (This adhesive, which is a single-package system, contains no solvent and cures by exposure to long wave ultraviolet light (320-400nm). It is suitable for fast precision bonding to glass, metal, and many plastics. Use it in any application that requires critical alignment or exact positioning.)

B. Operating procedure

I. Bending of glass capillary tubes

- Glass capillary tube is inserted into borehole of metal block (a centered, straight positioning should be achieved; use screw thread!) (**figure 1 A**, red arrow).
- Start melting/bending of glass capillary tube by heating the desired kink using a compressed gas can; most precise and straight bending is achieved when heating from above (vertical heating is ideal for eliminating unwanted sloped kinks).
- In an optimum way, bended glass capillary tube comprises a bending angle of exact 90° (**figure 1 B**), a straight bending without narrowing or squeezing diameter of glass capillary tube or angling the course of the glass capillary tube.
- Glass capillary tube needs to cool down before further processing!

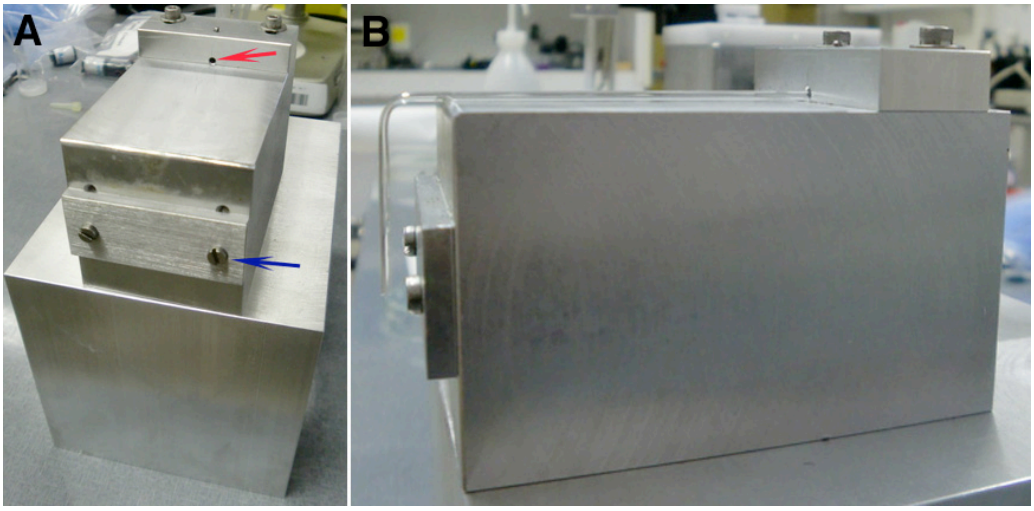


Figure 1. Bending of glass capillary tubes. A) Metal block for bending of glass capillary tubes to an angle of 90°. Borehole in the upper part, centered (red arrow), provides fixation of glass capillary tube (screw thread); centered metal plate in front (anchored via 2 screws, blue arrow) supports achieving an exact bending of the glass capillary tube to an optimum angle of 90° (B).

II. Attachment of hole-rings and meshes

- Long end of glass capillary tube is fixed into a borehole of the small metal block, which is connected to micrometer step motor (use screw thread) (**figure 2 B**); bended short end of glass capillary tube is pointing bottom-up first, to apply the adhesive.
- A tiny amount of adhesive is squeezed onto the surface of the glass Petri dish.
- Apply a tiny amount of adhesive to bended short end of glass capillary tube, exactly at the cutting site/cut surface; use any spiky metal item for applying the adhesive.
- Hole-ring needs to be exactly attached to the cut surface of the glass capillary tube; hole of hole-ring and hole of cut surface should match perfectly. (Caution: diameter of

glass capillary tube may not be narrowed by inclined/leaned hole-ring AND adhesive may not seal hole!). For attachment either the micrometer step motor plus supporting tip hole surface as ring-holder can be used, or attaching the hole-ring can be performed manually (**figure 2 A**, red arrow, and **figure 2 C**).

- For curing of adhesive capillary glass tube needs to be exposed to an UV light source for 1-2 min.

- Glass capillary tube needs to be readjusted into small metal block, connected to the micrometer step motor (using screw thread); bended short end of glass capillary tube points bottom-up for applying the adhesive.
- A tiny amount of adhesive is applied to the outmost edge of hole-ring (use any spiky metal item for applying adhesive). (Caution: adhesive may not contact hole of hole-ring, as in this case adhesive would seal mesh and interfere with low-pressure efficiency!).
- Mesh needs to be exactly attached to hole-ring; shapes of both rings should match perfectly! (Do not move mesh extensively; avoid spreading adhesive!).
- For curing of adhesive capillary glass tube needs to be exposed to an UV light source for 1-2 min.

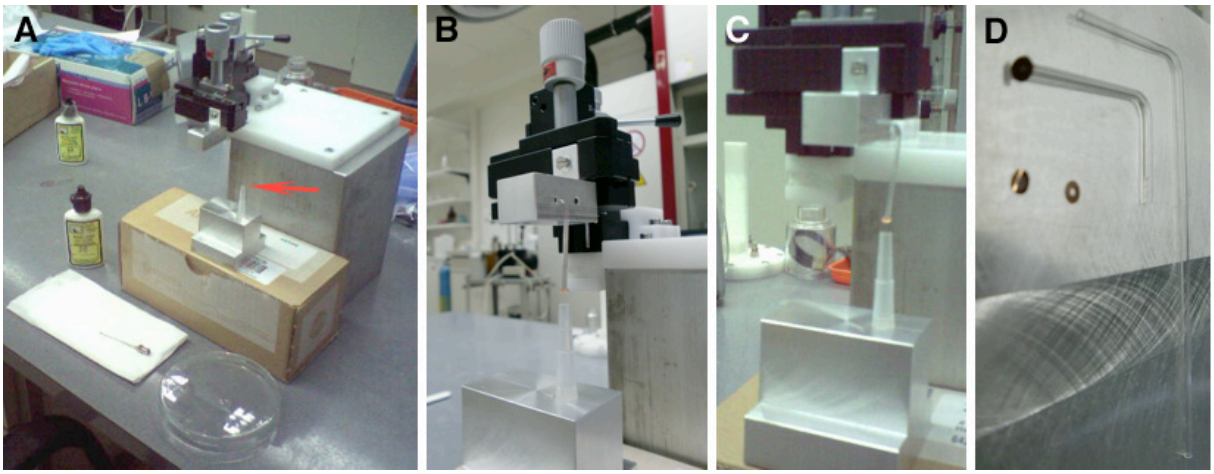


Figure 2. Working platform for applying hole-rings and meshes. A) Micrometer step motor fixed at a big metal block for XY-fine-positioning; small metal block with fitted supporting surface for attaching hole-rings and meshes (tip hole, arrow); UV curing adhesive; glass Petri dish as application platform for a tiny amount of adhesive; sharp/spiky item (metal tip) used for dosage and application of adhesive. B) Small metal block (fixed at the micrometer step motor) encompassing 2 boreholes for fixing the long end of bended glass capillary tube (screw thread); the short bended end of the glass capillary tube is facing the supporting surface (tip hole). C) Glass capillary tube after attaching hole-ring or mesh, respectively. D) Constituent parts of an adsorbing head device: glass capillary tube, hole-ring and mesh forming the sample take-up device.

9.1.2 LOP of LMD (laser microdissection) & SPATS transfer

"Transfer of microdissected material via low-pressure SPATS device"

A. Material

- New adsorbing head (glass tube, hole ring, lattice/grid according to **chapter 9.1.1**)
- Sample material fixed on PEN carrier membrane coated object slides (2 µm PEN-slides; MicroDissect GmbH, Herborn Germany)
- UVC light source (PCR Workstation, PeqLab Biotechnologie GmbH, Erlangen, Germany)
- Laser control box (CryLaS FTSS 355-50, CryLaS GmbH, Berlin, Germany)
- Softwares "Nanosauger 2.5/2.6/2.7" (XYZ High Precision, Darmstadt, Germany)
- Color firewire camera (PixeLINK, Megapixel Firewire Camera, BFI Optilas, Munich, Germany)
- Black & white CCD camera (Rolera-XR, QImaging, Surrey, BC, Canada)
- Softwares "QCapture" or "QCapture Pro 6.0" (QImaging, Surrey BC, Canada)
- Pressure-supplying pneumatic picopump (PLI-100 pressure control unit, Harvard Apparatus, Holliston, US)

B. Operating procedure

I. Preparations

- Switch on computer, microscope power supply box 231 and microscope button.
- Switch on laser control box → switch key to from "0" to "1", a green light appears (left side) → now the laser lamp is getting pre-warmed → wait until the second green light turns on (right side) → now the laser can be started by pressing the red button located between both green lights ("Laser On/Off") → when right green light turns to red, laser is ready to use.

II. Laser microdissection (LMD)

- Decontaminate adsorbing head by using a UVC light source.
- Make sure, that the arm of the transfer device is positioned out of the working area, on the right side of the XY-stage as a) this position favors an easy installation/fixation of the adsorbing head into the low-pressure supporting arm and b) the arm has enough space above to move up, as the software "Nanosauger" performs a calibration run of the XY-stage and the SPATS carrier arm when getting started.
- Either use the color firewire camera for colored images, or switch on the black & white CCD camera for black and white images.
- Run software "Nanosauger 2.5" (desktop) for fast working procedures (**figure 1**) and "Nanosauger 2.6" (desktop) for slow working procedures, both without using autofocus unit, or "Nanosauger 2.7" for working with implemented autofocus function (desktop). Softwares "Nanosauger" serve the color firewire camera, while for utilizing the black & white CCD camera additionally the software "QCapture" or "QCapture Pro 6.0" needs to be started.
- Optionally the stage movement direction can be adjusted via clicking "Control Via Buttons" and then clicking "Inverted X-Direction" as well as "Inverted Y-Direction"; re-activate the box "Control Via Joystick".
- Speed of stage movement and up/down-direction of SPATS arm can be set by clicking "Control Via Buttons", then the speed can be adjusted by moving the mode controller in the box "Speed" (settings 1-7); re-activate the box "Control Via Joystick".
- The SPATS arm can be move up/down by turning the joystick knob to the left and right; it can be moved left/right by pressing the arrow buttons in the box "Rotation", the speed of sideways movement can be adjusted by clicking "Low Speed", "Medium Speed" or "High Speed"; the length of the SPATS arm can be adjusted by turning the rotary knob located at the micrometer step motor.
- Test, if lattice is centered to the view field of the microscope by approaching the adsorbing head to the 10x objective; if not, center grid for easier sample take-up.
- For microdissection of single particles use the 40x objective; set microscope to "DL auf" (*Durchlicht* function is activated); laser can be switched on by operating the footswitch, or by activating the silver switch at the laser control box ("TRIGGER INT/EXT") to "INT" position; set microscope to "AL auf" (*Auflicht* function is

activated) calibrate the laser beam by focusing it onto the level of the PEN-carrier membrane, adjust laser focus and cut energy → a thin focused cut line is desired.

- Isolate single areas by keeping laser running and moving the microscope's XY-stage.
- To switch off laser, either loosen footswitch or turn silver switch ("TRIGGER INT/EXT") to "EXT" position.

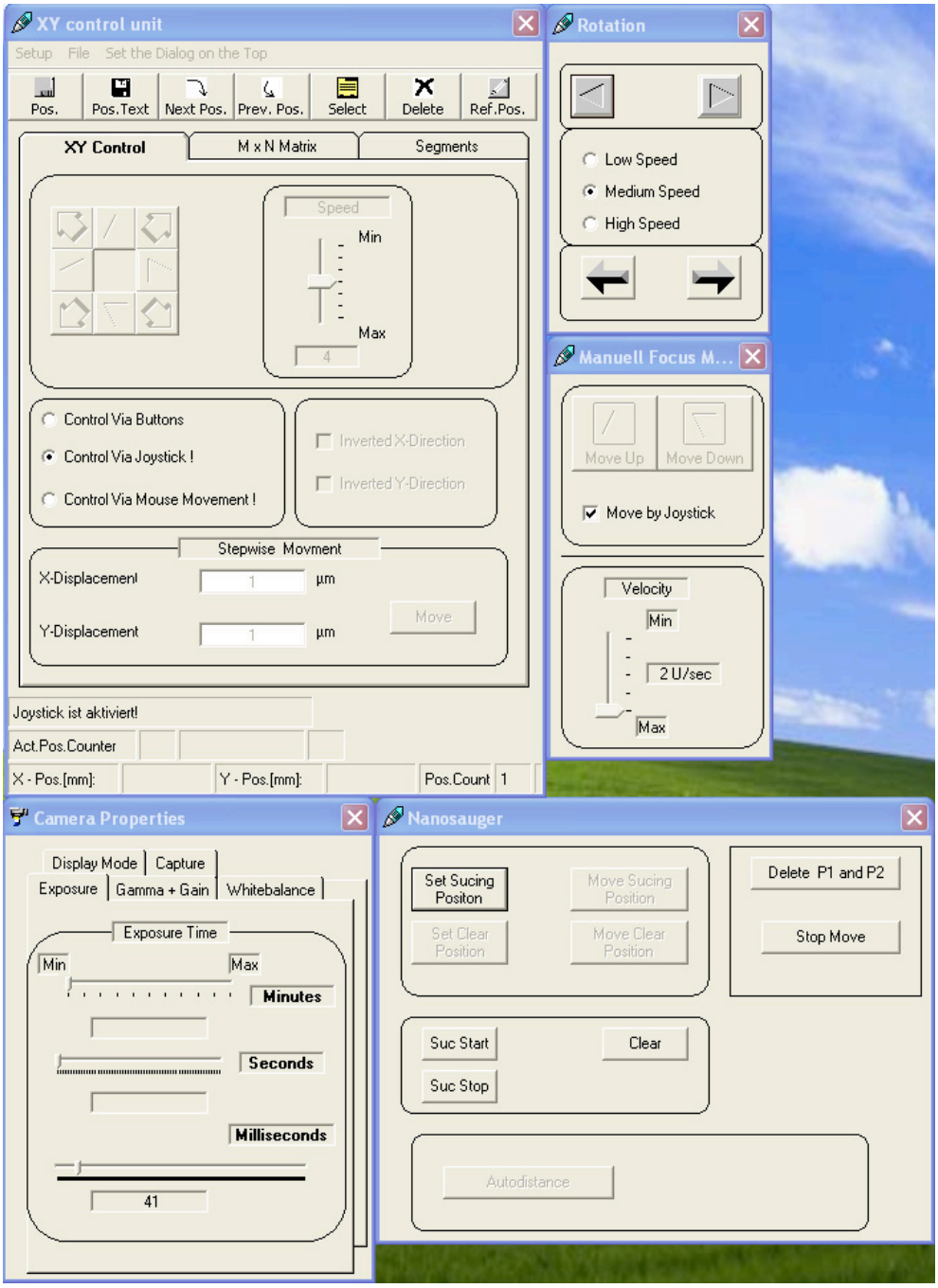


Figure 1. Screenshot of software "Nanosauger 2.5". Via the panel "XY Control unit" settings of the XY-stage can be changed. Activating the panel "Rotation" can move the SPATS device horizontally and various speed settings can be adjusted. The "Camera Properties" window allows taking pictures and provides several settings for optimized image taking. Via operating the "Nanosauger" panel, the low-pressure operation can be started (Suc Start/Suc Stop), stopped and a short impulse of high pressure can be applied by pressing "Clear".

III. Single particle adsorbing transfer system (SPATS)

- Use the 10x objective for controlled extraction/transfer via the SPATS device.
- Switch on pressure-supplying pneumatic picopump and turn on compressed air.
- Approach the grid/lattice of the adsorbing head to the surface of the isolated particle, start low-pressure process by clicking “Suck Start” (**figure 1**); check if particle is fixed to the grid and that the area of PEN-membrane is empty where isolation happened.
- Move SPATS up and transfer particle to a tube, planar device or else; release particle by pressing “Suck Stop” and “Clear” for providing a short impulse of high-pressure.
- Check particle release by having a look at the grid; for doing so, move adsorbing head back to the 10x objective and approach grid to objective lens.
- To quit operations, move SPATS arm out of the working area, to the right side of the XY-stage; remove adsorbing head and store it accurately; switch off microscope, switch off microscope power supply box, turn off laser by pressing the red button at laser control box (“Laser On/Off”), switch off laser control box via moving the key from “1” to “0” position, quit software, turn off camera, shut down computer.

9.1.3 LOP of LOC chips

"Application of LOC chips and of the LOC system"

A. Material

- LOC chips Cyto1, Cyto2, Cyto3 (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Ultrasonic cleaner (VWR International, Leuven, Belgium)
- UVC light source (PCR Workstation, PeqLab Biotechnologie GmbH, Erlangen, Germany)
- CytoCycler (PCR device incl. chip-holder, temperature control device, SAW control high frequency (HF) generator, particular software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany))
- HF generator (FC 1201 HF, Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Tickopur (DR. H. STAMM GmbH, Berlin, Germany)
- Acetone (Merck KGaA, Darmstadt, Germany)
- EtOH 100% (Merck KGaA, Darmstadt, Germany)
- PCR master mix (free of choice) and mineral oil cover (Sealing Solution, Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Sterile 0.2 ml PCR tubes (Eppendorf AG, Hamburg, Germany)

B. Cleaning of LOC chips (1-2x) in ultrasonic cleaner

- Clean chips with 70% EtOH, remove EtOH with paper towel (carefully tap the chip).
- Wash chips for about 5 min in 2% Tickopur solution using ultrasonic cleaner.
- Rinse chips thoroughly with H_2O^{dd}.
- Clean chips for 2-3 min in H_2O^{dd} (ultrasonic cleaner); blow dry with N_2.
- Clean chips for 2-3 min in acetone (ultrasonic cleaner); blow dry with N_2.
- Clean chips for 2-3 min in 100% EtOH (p.A. grade) (ultrasonic cleaner); blow dry with N_2.

- Incubate chips for 20 min using a UVC light source for decontamination.
- Successful cleaning can be tested by performing a negative control reaction on cleaned chips followed by detection via PAAGE ⇒ PCR should reveal no PCR product!

Important: as counting scale (graduation) at ultrasonic cleaner begins at "1" and not at "0" as written, add +1 min of time when starting the run (for 5 min put 6 min instead).

C. General information concerning LOC chips

- Currently, there are 4 different designs of LOC chips: Cyto1, Cyto2, Cyto2 plus microarray, Cyto3 plus microarray (**figure 1 C-E**).
- Cyto1 = original design, July 2007; hydrophilic reaction points A and B comprising a diameter of 40 µm and a hydrophilic track width of 15 µm (**figure 1 C**).
- Cyto2 = design for cell culture applications, January 2008; hydrophilic reaction point A comprises a larger diameter of 500 µm, instead of 40 µm as so far, for to let cells grow on the surface (the rest of the chip remained unchanged). Additionally, this chip charge was designed with a hydrophilic array field of 1x1 mm^2 next to reaction center B, for supporting microarray applications (**figure 1 D**).
- Cyto3 = totally new design, January 2009; hydrophilic track width was broadened to 200 µm instead of 15 µm as so far, especially concerning main track between reaction centers A and B (between transducer 10 and 5), and all tracks leading to reaction center B (between transducer 3 und 7); diameters of almost all reaction points (A, B and centers in front of IDT 3 and 7) are comprising 500 µm instead of 40 µm (red circles in **figure 1 E**); additionally, an intermittent hydrophilic circle is surrounding reaction point B to keep the oil cover more fixed to the surface (same design as used for the AmpliGrid™ AG480F, but with 4 gaps of 500 µm width). This chip charge was also designed with a hydrophilic array field of 1x1 mm^2 next to reaction center B, for supporting microarray applications.
- Spotting of oligonucleotide probes for microarray applications was performed using a "Nadelspotter" which can spot 100 µm spots, while the minimal distance between spots should be 50 µm. The 1x1 mm^2 array field (reaction point C) comprises dimensions of 1x1 mm^2, thus 36 spots can be arranged in a 6x6 array. Spotting can also be performed in reaction spot B, while there only a 3x3 array is possible due to smaller diameter.

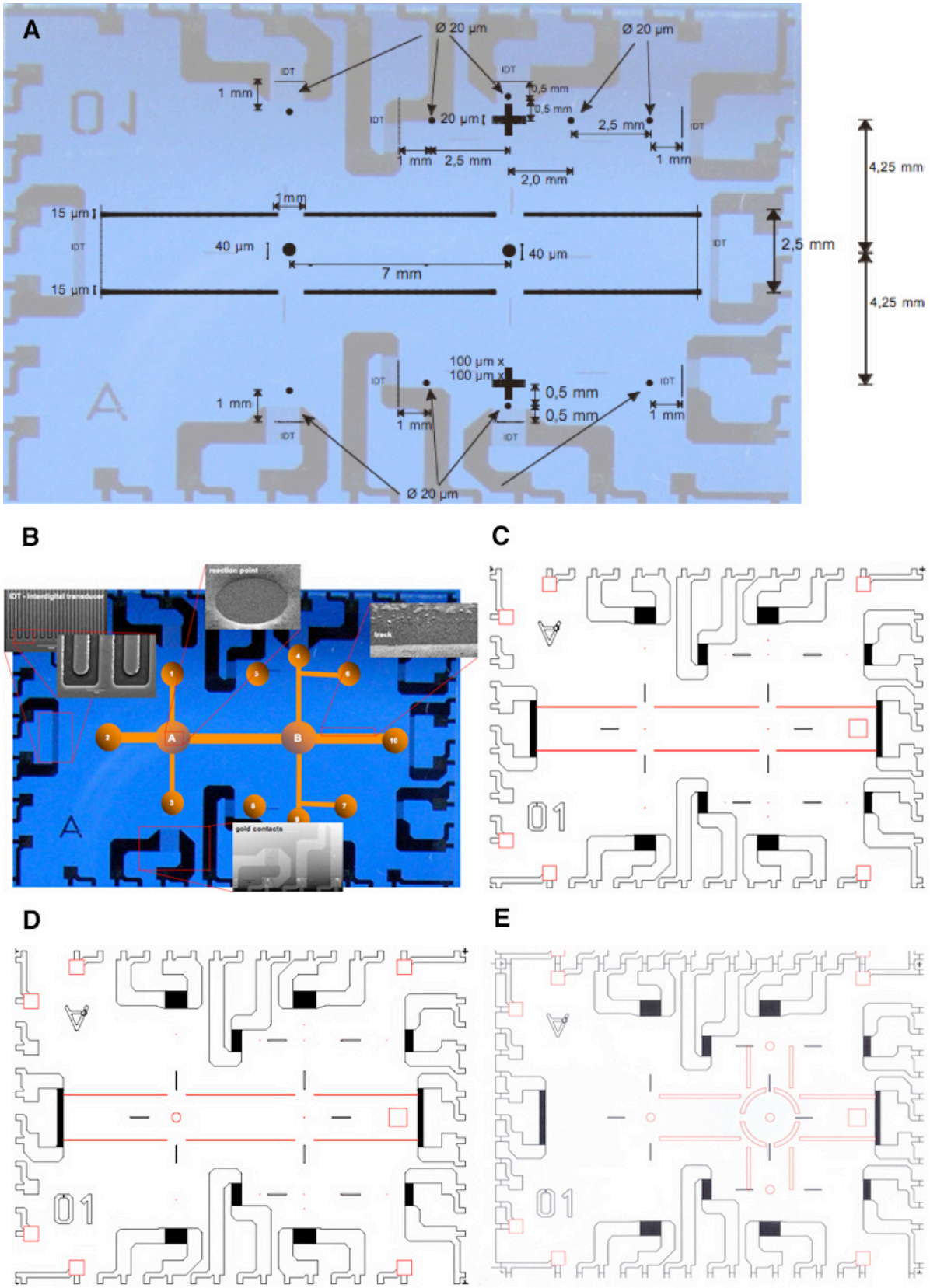


Figure 1. Design of LOC chips. A) Design and dimensions of chip charge Cyto1. B) Electromicroscopy images of hydrophobic/hydrophilic track system and reaction points. C) Layout of Cyto1: ∅ point A 40 µm, ∅ point B 40 µm, track width 15 µm. D) Layout of Cyto2, with microarray: ∅ point A 500 µm, ∅ point B 40 µm, 1x1 mm array field (point C), track width 15 µm. E) Layout of Cyto3 with microarray: ∅ point A 500 µm, ∅ point B 500 µm, 1x1 mm array field (point C), track width of red lined tracks 200 µm.

- Chips were manufacturer and provided by Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany; spotting operations were done there as well.
- The basic material of each chip is $LiNbO_3$. Each chip comprises 10 interdigital transducers (IDTs) with service connections and gold contact pads. Big IDTs for serving the main track should provide an aperture of 3 mm to be capable of moving 5 µl of oil, the smaller ones for moving droplets an aperture of 1 mm. There must be at least 4 different SAW frequencies to operate 4 IDTs at a time. A homogeneous passivation using SiO_2 enables chemical modification. The surface chemistry features hydrophobic and hydrophilic areas. Hydrophilic areas provide tracks for controlled droplet movement by surface acoustic wave operation (SAW) and distanced reaction centers, which enable independent temperature control.

D. Installation of LOC chips into the CytoCycler device and setting frequencies/channels at the FC 1201 HF generator for the particular chip charge

- The FC 1201 HF generator distributes according to the joystick settings high frequency signals in the range from 120-170 MHz to one of four channels. The maximal power/capacity comprises 35 dBm and is controlled continuously from 7 dBm to maximum via the deflection of the joystick. The HF generator has 3 different switching-status options, marked via grouping into A, B and C (**figure 2 A**). The particular switching status is displayed in the upper middle of the front panel (**figure 2 A**, blue arrow). States can be switched by tripping the left joystick button. By tripping the right joystick button, the generator switches to maximum power in the direction the joystick is moved along the main track. By pressing the “fire” button on top of the joystick, maximum power in the appropriated direction is provided, while the maximum corresponds to the set value. Each switching status operates 4 different HF channels and connected SAW transducers by moving the joystick (**figure 2 A**, red arrow). The relation of each transducer to the single groups in shown in **figure 2 C**.
- When installing chips into the chip-holder of the CytoCycler, the temperature control device must be switched off – otherwise the temperature sensor and the whole device will get damaged! Additionally, the HF generator (SAW control box) may not be run without a chip installed! (HF channels may not be run with “open end” respectively without connected SAW LOC chip).

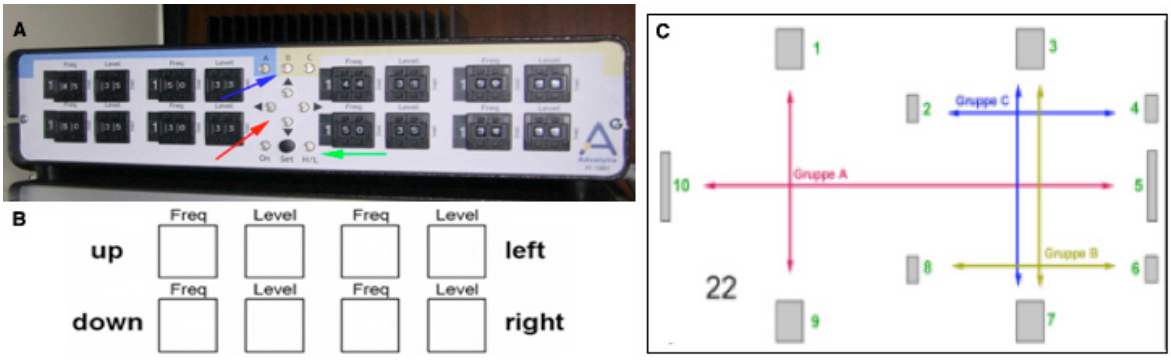


Figure 2. Operations on a LOC chip. A) Switching-status options A, B and C displayed at the panel (blue arrow). Joystick deflection shows operating HF channels (A, red arrow and B). C) The image shows the relation of transducers and switching status grouping. The arrows display the movement capacities/facilities of a droplet under the influence of the transducers of this group.

- For installing a chip, the contacting lid for the high frequency support of the chip-holder is opened by turning both screws and release the click-fastening mechanism (**figure 3**, red arrows); carefully remove SAW control lid and put in a chip preferably fixed to the left corner of the cavity for being optimally connected to IDTs by gold contacts and gold contact pins.

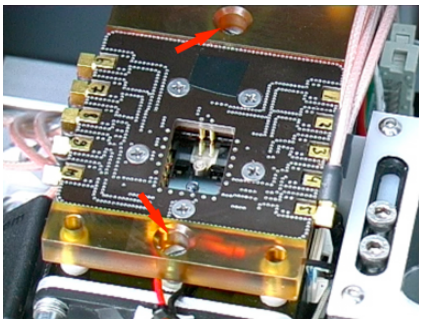


Figure 3. Installation of LOC chips. Chips are inserted into the chip-holder via screw-operated "click-fastening mechanism".

- Re-attach SAW control lid and fasten screws.
- For each chip-charge, a report is provided telling the numbers of the chips, the number of single defect transducers as well as dedicated frequencies/channels for being tuned at the HF generator.
- Those frequencies/channels and the power level then need to be set at the front panel of the HF generator due to the following scheme (**figure 2 A**): the four control elements on the left side are valid for area A on the chip, while the four control elements on the right side are valid for area B as well as area C. Always 4 transducers must be operable by joystick at a time. So an 8-channel HF generator is used, whereby

2 channels at a time should provide the same frequency. The joystick provides a switch to shift between the first 4 and the second 4 channels. The HF generator provides a PC-interface to enable software-controlled droplet movement. Thereby the upper left control element represents values for "up", the lower left one for "down", upper right one for "left" and the lower right one for "right" (**figure 2 B**); given directions are related to the tracks in the respective area in which the droplets are moved according to the acting IDTs. Thus, directions display the agitation facilities of a droplet being under the influence of these interdigital transducers. Currently droplet movement can only be carried out manually via joystick, but not yet via software. Important: changed values need to be saved, validated and transferred to the SAW control unit by activating the "Set" button. For details about the connection of IDTs and 1201 HF generator please refer to the "FC 1201 HF-Generator Manual" provided by Zeno Guttenberg (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany) in 2006. Important: in case of a non-working joystick (shown by blinking lights at the front panel), the joystick needs to be re-adjusted. This can be done by opening the joystick covering at the rear side and measuring the co-current flow of the contacts for joystick deflections. The measured value should be set around 2.5 V.

- The chip-holder is sometimes also named "CytoCycler". It has a cavity for installing a single chip. The contacting lid for the high frequency support, which must be removed and reassembled for installing a chip, provides power connections for 10 transducers including 20 gold contact pins.

E. Operating the CytoCycler for performing PCR

- Install a new chip in the LOC chip-holder; important: the temperature control device may only be switched on when a chip is inserted – otherwise it might be damaged!
- Start computer, switch on temperature control device (black heating device), run software "CytoCycler" (desktop). For a detailed description of the software and the different program cards please refer to the "CytoCycler Software Manual" provided by Zeno Guttenberg in 2006 (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany).
- The software provides different register cards, where programs for the load resistor heating (serving reaction point A) as well as the Peltier element (serving reaction point B and the array field C) can be saved; required register cards can be activated/marked

in the box “Status” (working procedure then happens from A1 to B3); for performing a normal PCR, simply register card B2 is required, where a complete PCR program can be typed in, saved and changed via “Save” or “Save as”. Saved programs can be changed and activated by “Load”→ “All Files“ → choose program. Single boxes in the cards, where the duration was set as “0” s, are going to be ignored.

- Load resistor heating accepts temperatures from 20°(room temperature)-90°C, with a heating rate of 0.01-10 K/s; duration of temperature steps can be set to up to 16 h (60000 sec). The Peltier element accepts temperatures from 4°-105°C, with a heating/cooling rate of 0.01-5 K/s (cards A1, A2, B1) and a fixed heating rate of 3 K/s and cooling rate of 4 K/s. Cycling times can be chosen up to 200 s per temperature step, and times for pre-annealing or post-elongation up to 3000 s. Temperature increments can be set from 0.1-5 K, and time increments from 0.1-5 sec. The number of cycles can be adjusted from 0-60.
- Load a saved program file: “Load” → “All Files” → choose program; parameters can be changed individually; run program by clicking “Start Process”; after chosen parameters have been checked by the software, a message box appears (“B2 Kommentar”) to definitely start the reaction by clicking “Ok”; each program can be stopped by clicking “Abort Process”.
- Before starting a PCR, each new chip which is installed into the chip device, needs to be pre-heated to 95°C for about 15 min C (during this procedure, the material of the chip expands due to the high temperature – this prevents the droplet afterwards, when starting the initial denaturation step of the PCR at 95°C, to be moved/deformed due to material stress); afterwards, let the chip cool down completely.
- For performing PCR, first, add 1 µl of master mix onto reaction point B, then cover the reaction by adding 5 µl of mineral oil (Sealing Solution) to prevent evaporation and cross-contamination. Evaporation might inhibit the amplification reaction, as via evaporation the salt concentration of the reaction solution gets enhanced.
- Start PCR by “Start Process”.
- Let chip CytoCycler cool down after PCR is finished (wait until ventilation stops).
- Transfer 1 µl PCR into a sterile 0.2 ml PCR tube for storage (e.g. add 1 µl of 6x gel-loading-dye as well as 4 µl of water, then extract 6 µl volume from PCR tube for PAAGE application).

- Clean used LOC chips immediately with 70% EtOH and H_2O^{dd}, then store in water for a distinct period of time to remove dried PCR remains; continue with cleaning procedure (paragraph A of this LOP).
- Insert a "dummy chip" into the cavity of the chip-holder serving as a placeholder, as CytoCycler device may never be assembled without a chip inside!!

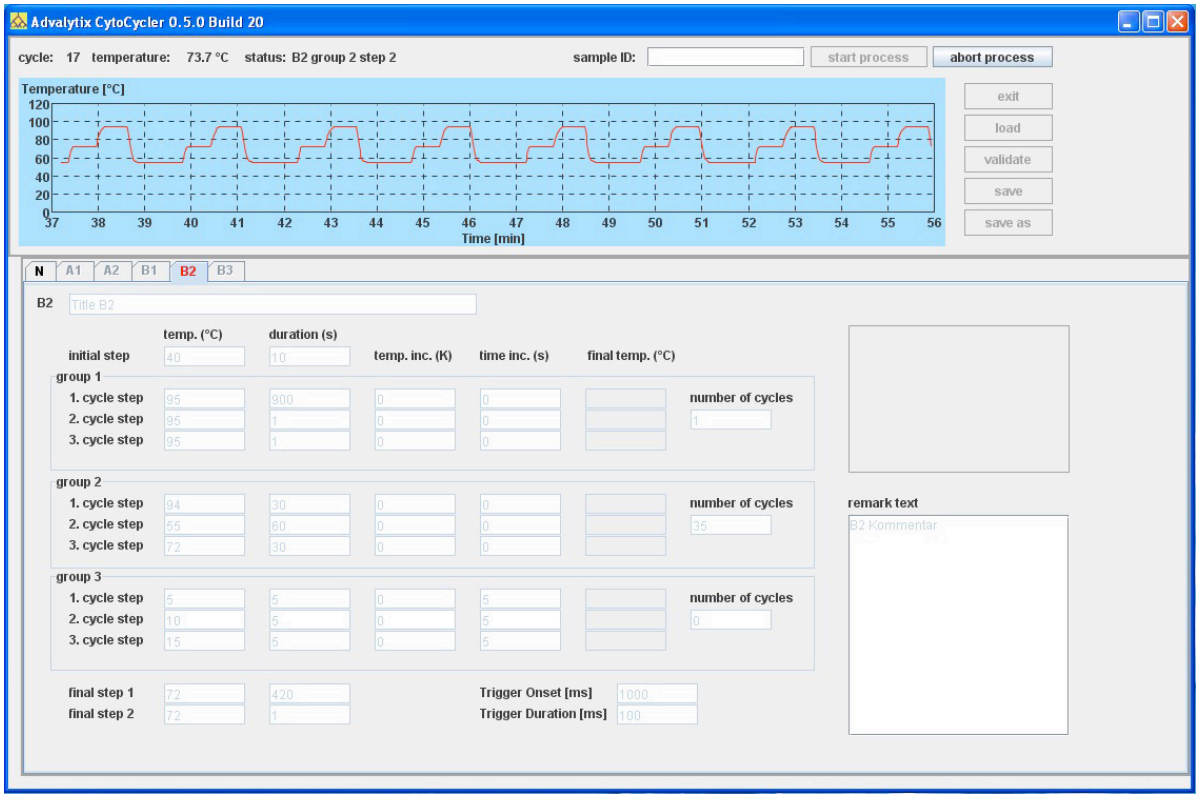


Figure 4. Screenshot of software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany**).** Several working sheets N-B3 comprising various PCR programs and protocols are independently addressable and operable. In the upper left corner a steady overview is provided concerning cycle number, temperature and activated working sheet. The temperature profile of the passed 20 minutes is shown graphically as well.

9.1.4 LOP of LV-PCR (low-volume PCR)

"Low-volume (LV-PCR) for application on AmpliGrid™ AG480F and the LOC system"

A. Material

- AmpliGrid™ AG480F or LOC chips Cyto1, Cyto2, Cyto3 (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- AmpliSpeed slide cycler (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- UVC light source (PCR Workstation, PeqLab Biotechnologie GmbH, Erlangen, Germany)
- CytoCycler (PCR device incl. chip-holder, temperature control device, SAW control high frequency (HF) generator, particular software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany))
- HF generator (FC 1201 HF, Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Sealing Solution (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany)
- Sterile 0.2 ml PCR tubes (Eppendorf AG, Hamburg, Germany)
- Purified DNA material or crude sample material for direct cell lysis, but not more than 1 ng target DNA material – this might overload the reaction volume!
- Master mix (MM) for performing PCR including buffer for DNA polymerase, dNTPs, $MgCl_2$, oligonucleotids (primers), DNA polymerase: it is important to use an appropriate MM, which DOES NOT mix/fuse with the cover oil (Sealing Solution)!! Most suitable turned out to be PCR kits provided by QIAGEN GmbH (Hilden, Germany) like e.g. QIAGEN® Fast Cycling PCR kit; QIAGEN® Multiplex PCR kit; QuantiFast™ SYBR® Green I PCR kit; QuantiTect™ SYBR® Green I PCR kit).
- Master mixes need to be calculated down to exactly 1 µl of total reaction volume per reaction; there are two possibilities: a) calculate master mixes (w/o DNA) to 1 µl per reaction point excluding volume of target DNA, as target DNA or the tissue

sample/cell respectively has already been placed at the reaction point and dried at room temperature; b) master mixes are calculated to 1 µl per reaction including volume of target DNA (standard procedure). Caution: drying sample material at room temperature on LOC chips worked always suboptimal and caused a lot of bubbling master mixes when applied to the sample; thus, just procedure b) should be used for LOC chips. On the AmpliGrid™ AG480F, both procedures worked comparable well!

- AmpliGrid™ AG480F PCR: all cycling times for PCR need to be extended a bit, as slide cycler needs some time to reach the desired temperature (each temperature step about 20 sec longer); use higher concentration of polymerase (1U/µl); some kits perform better when scaled down, others worse (best: PCR kits by QIAGEN GmbH can be scaled down 1:1); primer concentration about 0.2 µM in PCR; use a DNA starting amount of about 100-200 pg, 1 ng at maximum; concentration of Mg^{2+} about 1-5 mM; initial denaturation step as well as final elongation step each about 5-10 min.
- 1 µl of PCR reaction master mix is generally covered with 5.0 µl of Sealing Solution. When using microdissected material, the PEN-carrier membrane might interfere amplification; thus, time of initial denaturation could be extended to 15 min. When droplets next to each other merge, less oil needs to be used (4.8-5.0 µl) (**figure 1**).

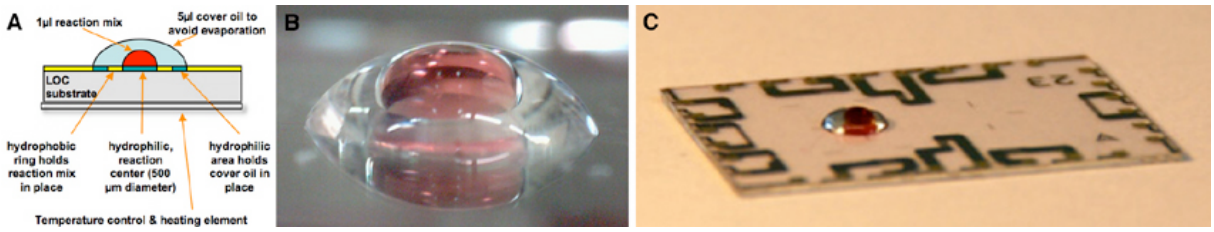


Figure 1. Principle of low-volume PCR (LV-PCR). A) Schematic overview: 1 µl of hydrophilic master mix is placed on a hydrophilic reaction spot and kept in place by a surrounding hydrophobic ring; hydrophobic oil coverage prevents evaporation and is kept in place by a surrounding hydrophilic ring. B) Close-up of the droplet-in-oil principle of LV-PCR on chip. C) LOC chip presenting a placed PCR reaction, comprising 1 µl droplet of master mix (red colored as containing blood) covered by 5 µl of mineral oil.

B. Operating procedure

I. Preparing the master mix

- Clean room (use overshoes, gloves, lab coat): all PCR reagents are stored at -20°C in the freezer, EXCEPTING DNA!!!
- 1 µl LV-PCR master mix for β-actin or amelogenin PCR respectively (QIAGEN® Fast Cycling PCR kit): 0.5 µl 2x QIAGEN Fast Cycling PCR Master Mix (final 1x), 0.1 µl

10 µM forward primer (final 1 µM), 0.1 µl 10 µM reverse primer (final 1 µM), 0.0-0.3 µl sterile water (Ampuwa®, Fresenius GmbH, Bad Homburg, Germany) (depends on DNA amount/volume used or if DNA was dried on chip). Amelogenin primers (stock solution 100 pmol/µl) = 'Amel1' (5'-CCC-TGG-GCT-CTG-TAA-AGA-ATA-GTG-3') and 'Amel2' (5'-ATC-AGA-GCT-TAA-ACT-GGG-AAG-CTG-3'); β-actin primers (stock solution 100 pmol/µl) = 'β-Actin up' (5'-TCA-CCC-ACA-CTG-TGC-CCC-ATC-TAC-GA-3') and 'β-Actin down' (5'-CAG-CGG-AAC-CGC-TCA-TTG-CCA-ATG-G-3').

- Calculate at least 1 negative control (just MM w/o DNA) and 1 extra reaction per reaction batch (as reserve, that there will be enough master mix for all reactions – note, that there are pipetting inaccuracies due to natural measurement error of pipets and MM sticking to filter tips and tubes!).
- Important: mix reactants of master mix just by ‚agitating/stirring the pipet', DON'T mix up and down!!! Extensive mixing might create smallest air bubbles in the master mix, which could lead to the generation of big bubbles in the reaction mix while heated to 95°C (Loss of reactants! Loss of volume! Change of concentrations!).

II. Setting up a LV-PCR

- Decontaminate AmpliGrid™ AG480F and LOC chips for 15-20 min using a UVC light source.
- Position negative control first and then add DNA to the mix (in case that DNA has not been air dried at reaction points).
- Add 1 µl MM per reaction point (volume can even be scaled down to 200 nl [1]), then cover droplets by using 4.8-5.1 µl of Sealing Solution to prevent evaporation and cross-contamination (**figure 1**). Evaporation might inhibit the amplification reaction, as via evaporation the salt concentration of the reaction solution gets enhanced.
- Important item when pipetting: empty pipet just until the first pressure point is reached!!! Emptying pipet down to the second pressure point can cause bubble formation in maser mix and Sealing Solution due to the additionally blown off dead volume (enhanced pressure)!!

III. Standard PCR programs

- β-actin and amelogenin PCR: 5-15 min at 95°C initial denaturation (depends on MM and required activation time of HotStart DNA polymerase), 35-40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 1 min (cycling times and temperatures can be adjusted individually).
- "Touch down PCR" for DNA typing using primers D7S1824, D9S302, D10S2325: 10-15 min at 95°C initial denaturation (depends on MM and required activation time of HotStart DNA polymerase), 14 cycles of 94°C for 30 sec, 64°-50°C for 60 sec (temperature increment -1°C per cycle), 72°C for 30 sec, followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, final extension at 72°C for 7 min.
- AmpF/STR® SEfiler™ PCR amplification system (Applied Biosystems, Darmstadt, Germany): 11 min at 95°C initial denaturation for HotStart AmpliTaq Gold® DNA polymerase (5-15 min, depends on MM and required activation time of HotStart DNA polymerase), 28 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, followed by 60°C for 45 min final extension (for AmpliTaq Gold® DNA Polymerase mediated non-template adenylation) or respectively 15 min (for KOD Xtreme™ DNA Polymerase = blunt ends, normal extension).

C. Operating the AmpliSpeed (LV-PCR on AmpliGrid™ AG480F)

- Switch on AmpliSpeed slide cycler; log in as 'Administrator'; wait until self-test and calibration is done (display changes from "Self test" to "Idle").
- Load a saved program file: "File" → "Load" → "PROTOKOLLE" → choose program → click "Ch. Dir", which stands for "ok".
- For changing some parameters of the selected program: address/click on the parameter (e.g. time, temperature or cycle number) using the touch screen, change settings using the arrow buttons; when starting the changed program, automatically the software calls for saving the changed program – this can be confirmed, otherwise the changed program can be save using a new file name.
- Position the AmpliGrid™ AG480F onto the heating area of the AmpliSpeed slide cycler and close the lid.
- Run program by activating the "Start" button (arrow button).
- After PCR has finished, stop program by clicking the "Stop" button (square button).

D. Operating the chip system (LV-PCR on CytoCycler)

- Install a new chip in the LOC chip-holder (important: the temperature control device may only be switched on when a chip is inserted – otherwise it might be damaged) (see detailed description in the "LOP of LOC chips", **chapter 9.1.3**).
- Start computer, switch on black temperature control box, run software "CytoCycler" (desktop).
- Load a saved program file: "Load" → "All Files" → choose program; parameters can be changed individually; run program by clicking "Start Process"; after chosen parameters have been checked by the software, a message box appears ("B2 Kommentar") to definitely start the reaction by clicking "Ok"; each program can be stopped by clicking "Abort Process".
- Before starting a PCR, each new chip which is installed into the chip-holder, needs to be pre-heated to 95°C for about 15 min C (during this procedure, the material of the chip expands due to the high temperature – this prevents the droplet afterwards, when starting the initial denaturation step of the PCR, to be moved/deformed due to material stress); afterwards, let the chip cool down completely.
- First, add 1 µl of master mix onto reaction point B, then cover the reaction by adding 5 µl of mineral oil (Sealing Solution) to prevent evaporation and cross-contamination (**figure 1**). Start PCR.
- Let chip CytoCycler cool down after PCR is finished (wait until ventilation stops).
- Transfer 1 µl PCR into a 0.2 ml sterile PCR tube for storage (e.g. add 1 µl of 6x gel-loading-dye as well as 4 µl of water, then extract 6 µl volume for PAAGE applications).
- Clean used LOC chips immediately with 70% EtOH and H_2O^{dd}, then store in water for a distinct period of time to remove dried PCR remains; continue with cleaning procedure (see "LOP of LOC-chips", **chapter 9.1.3**).
- Insert a "dummy chip" into the cavity of the chip-holder serving as a placeholder, as CytoCycler device may never be assembled without a chip inside.

9.1.5 LOP of BioSpot® (PipeJet™)

"Application of BioSpot® for automatic spotting of solutions onto the LOC"

A. Material

- BioSpot® dispenser and software "BioSpot®" (BioFluidix GmbH, Freiburg, Germany)
- 0.2 ml sterile PCR tubes (Eppendorf AG, Hamburg, Germany) used as reservoirs/flasks for fluids (e.g. master mix, Sealing Solution, SSC washing solutions after hybridization/array amplification, etc...)
- e.g. master mix solution (without DNA, to not contaminate tips/PipeJets™ of BioSpot®), filled into 0.2 ml Eppendorf reaction tubes, for reaction center A or B
- Sealing Solution (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany), filled into 0.2 ml Eppendorf reaction tubes, for reaction point C
- e.g. washing solutions (various concentrations of SSC), filled into 0.2 ml Eppendorf reaction tubes, for reaction center A or B

B. Protocols

I. Starting the Biospot® (Nanodispenser)

- Start computer, switch on hardware (piezo control unit & movement control), start software "BioSpot®" (drive C:\\Program Files/BioSpot/software/BioSpot.exe or via shortcut "BioSpot®" on desktop) → at first, automatically LOC-slide and PipeJets™ move to parking position (reference position) at x=0 or z=0 respectively (initial position).
- Adjust speed/velocity of x- and z-axis in control window "Axis Control": Xvel = 500, Zvel = 500, press "Set Axis Speed" to validate changed settings; adjust the trackbar below for "Step width (mm)" to smallest values as well (= 0.01 mm) ⇒ by adjusting these settings the motor gets prohibited of slippage/wheelspin when movement settings for x-position are changed from 0 to increasing values. In case of wheelspin, recalibrate the system by "Search Reference".

II. Description of single active control windows (**figure 1**)

- Window **"(1) Axis Control and Axis Movement"**: for movement of LOC-slide on x-axis and of PipeJets™ on z-axis. For manual handling just the following settings are needed: "Move to Pos" for moving the LOC-slide and PipeJets™ in desired positions on the x- or z-axis respectively. Simply set desired numbers and start application with "Move" (black arrow buttons can only be used for movement when window "Enable Keyboard Control" is activated, but are not needed necessarily. Buttons "Search Reference", "Stop Search" and "Move to Parking Position" (x = 0, z = 0) are not needed for normal application as well). All of the following values given in **table 1** are valid for 0.2 ml Eppendorf reaction tubes, set into the various flasks. Initially 1.5 ml flasks are set into the desired position A, B, C or D of the flask holder, into there 0.5 ml reaction tubes are set, and into there, finally 0.2 ml reaction tubes. That "tube-in-tube"-application is used to keep applied pipetting volumes as small as possible, to get PipeJets™ deeper into tubes and to not waste much fluid just for filling up the big 1.5 ml tubes. Standard values for x- and x-movement are give in **table 1**.

Table 1. Functions and values for using the BioSpot®.

LOC application	**Position x-axis [mm]**	**Position z-axis [mm]**
Aspiration PJ1 (flask A)	280	33-37
Aspiration PJ1 (flask B)	266.5	33-37
Aspiration PJ1 (flask C) (best centered)	253.5	33-37
Aspiration PJ1 (flask D)	240.05	33-37
Aspiration PJ2 (flask A)	280	33-37
Aspiration PJ2 (flask B)	266.5	33-37
Aspiration PJ2 (flask C) (best centered)	253.5	33-37
Aspiration PJ2 (flask D)	240.05	33-37
Aspiration PJ3 (flask A) (best centered)	280	33-37
Aspiration PJ3 (flask B)	266.5	33-37
Aspiration with PJ3 using flask C and D is not applicable due to steric interference with cable/wire below the flask holder		
Dispensing PJ1 to reaction center A (H_2O)	62 - 62.5	30
Dispensing PJ2 to reaction center B (H_2O)	114.2 (115)	30 (28)
Dispensing PJ3 to reaction center C (oil)	164-166	25-28
To check, if tip of PipeJet™ PJ1, PJ2 or PJ3 is centered to flasks A, B, C or D	Flask position	28
SPATS Position (for sample release)	700	-
Fluorescence Reader	459 - 460	-

- Window **“(3) Valve Control and Pump Control”**: to open and close valves of single PipeJets™ and to connect pump with valves, or with air or valves with air (bypass). The valve, which is going to be used to “Aspirate”, must be open and connected to the pump (button “Valves (2)”), the other valves must be closed. The speed „PSpeed“ for „Aspirate“ or „Dispense“ respectively can remain unchanged at 20 ms. The volume „PVol“ can be adjusted: e.g. to 30 µl for H_2O, master mix and washing solution (PJ1 + PJ2), to 40 µl for oil (PJ3). After „Aspirate“ valves can remain connected to the pump, avoiding a low-pressure to occur. The “Dispense” function is needed only to totally empty the valves and to dispense remaining liquids back into the flasks.
- Window **“(2) PipeJet™ Control”**: various settings for automatic shooting of a definite volume using single PipeJets™. There are just slight changes to be validated as most parameters are set as standard values and remain unchanged. Standard settings: Istroke = 36 µm, vdown = 200 µm/ms, thold = 10 µs, vup = 2 µm/ms, n = 5, delay = 100 ms. Just the “Istroke“ is recommended to be changed to 20 µm instead of 36 µm, and the number “n“ from 5 to 20 repetitions, when 1 µl is going to be shot. Any changes in the settings need to be validated by “Set PJ“. A volume of about 1 µl is shot, when having aspirated a volume of about 30 µl with PJ1 or PJ2 and when starting the shoot-function by “Shoot PJ“. Buttons “Detect mode“ as well as „Dispense mode“ do not have a specific function defined by the software and can be neglected.
- Window **“(4) Batch Mode”**: for programming and saving complex operations (in an excel-sheet). Thereby, a numerical code is applied. In the control window „(1) Axis Control and Movement“ x-position values indicate a movement of the LOC-slide, z-position values indicate a movement of the PipeJets™. In the control window „(3) Valve and Pump Control“ for valves V1, V2, V3 a “0“ indicates open valves and a “1“ means closed ones. For using the pump, at “PPos” a “0“ stands for “Bypass”, that is the connection of „Valve with Air“, “1“ indicates a connection “Pump with Air“ and “2“ means the connection “Pump with Valves“. Volumes for “Aspirate“ are indicated at “PVol“ using a “-“ for negative values, volumes for “Dispense“ using a “+” for positive values. Errors during the operation can be checked and detected by “Check Batch“. The operating process can be started with “Batch execute“ and can be paused with “Pause” or aborted with “Stop execution”. When the “Stop execution” function is activated, the total batch process stops immediately and the execution is cancelled. When pressing “Batch execute” again, the system will try to get back to the initial

starting positions of the actual batch file (the first line), and x-axis as well as z-axis will move simultaneously. Thus, to avoid PipeJets™ hitting the LOC-slide, use the "(1) Axis Control and Axis Movement" box to get the PipeJets™ (firstly) as well as the LOC-slide (secondly) manually back to the initial positions at z=0 and x=0, before starting "Batch execute" again. When "Pause" is pressed, a window "Batch Processing Paused" will appear. Now it is possible to change settings manually in window (1), (2) and (3), and to perform extra performances. However, the system will be back to the actual position in the batch mode and continue, after pressing "Ok". So click "Ok" to continue.

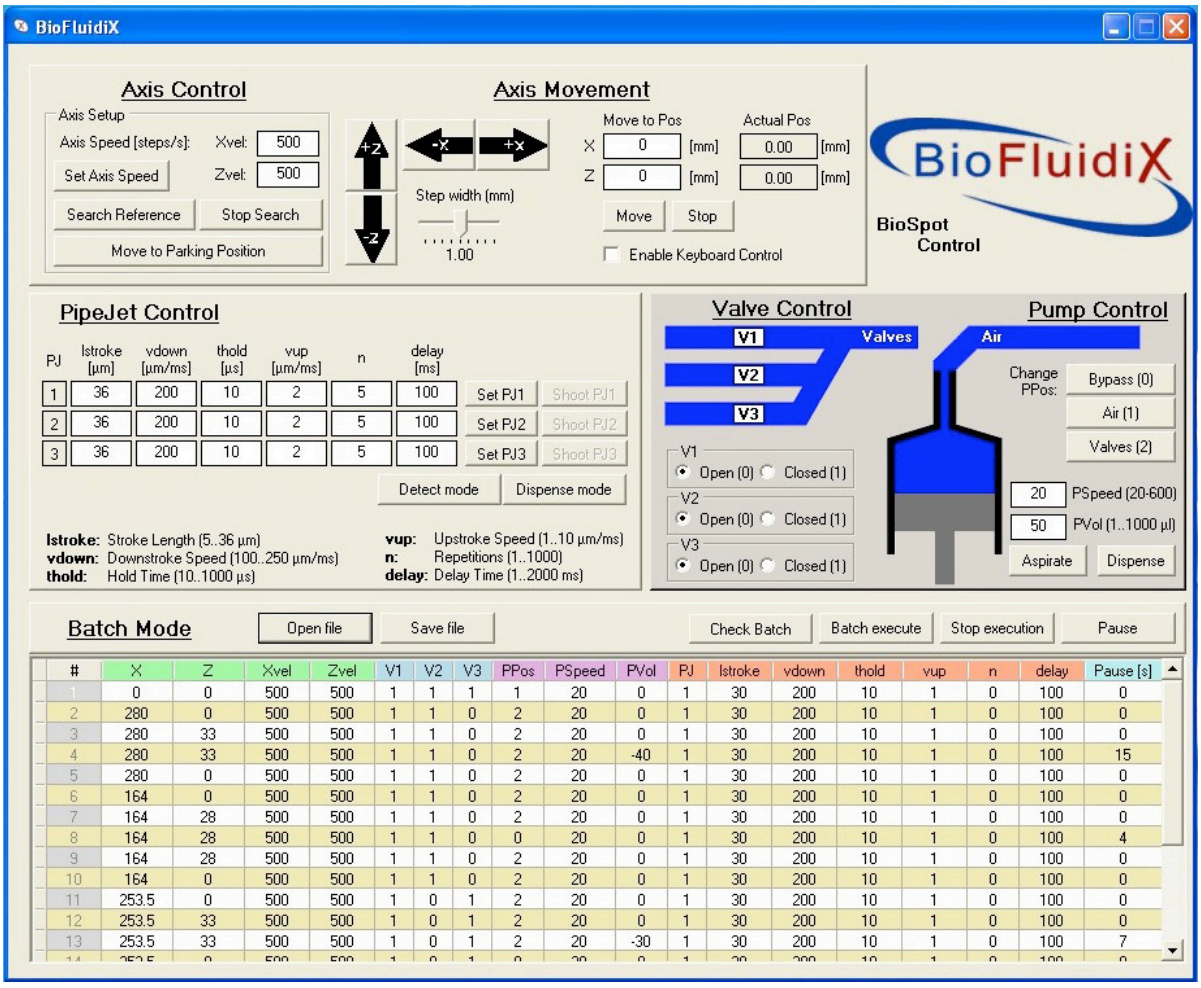


Figure 1. Screenshot of software "BioSpot®" (BioFluidix GmbH, Freiburg, Germany). The software is used for operating the automatic dispensing device BioSpot®. There are four single active control windows namely "(1) Axis Control and Axis Movement", "(2) PipeJet™ Control", "(3) Valve Control and Pump Control" for manually operated applications, as well as "(4) Batch Mode" for automatically operating protocols. (1) allows a regulation of speed and x-position of the slide rail and z-position of PipeJets™. (2) allows an adaption of depth of penetration, displacement speed, release speed and holding time of the piezostack driven piston, as well as number of repetitions and delay time. (3) allows opening and closing of PipeJet™ valves, connections to the syringe pump as well as setting speed and volumes for aspiration and dispension and to start these operations. (4) allows programming complete sequences, which can be run automatically.

III. Manually performed spotting of 1µl of master mix onto reaction point A (PipeJet™1,PJ1)

- (1) “Move” LOC-slide to x-position 253.5 = flask C; “Move” PJ1 to z-position 33-37.
- (3) “Connect” pump to valves, “Open” valve1, “Close” valves 2 and 3; PSpeed = 20, PVol = 30 µl; press button „Aspirate“ (watch, if fluid is aspirated into the tube); pump stays connected to valve1.
- (1) “Move” PJ1 to z-position 0; “Move” LOC-slide to x-position 62 = reaction center A; “Move” PJ1 to z-position 30.
- (2) Adjust the following settings at PJ1: Istroke = 20 µm, vdown = 200 µm/ms, thold = 10 µs, vup = 2 µm/ms, n = 20, delay = 100 ms; validate settings with „Set PJ1“; start spotting with “Shoot PJ1“; about 1 µl will be spotted onto the chip surface.
- (3) After shooting, “Close” valve1 and “Connect” pump to air.
- (1) “Move” PJ1 to z-position 0.
- SAW-Control: 1 µl of fluid can now be move via SAW on the chip surface.
- (1) “Move” LOC-slide to x-position 253.5; “Move” PJ1 to z-position 28-30.
- (3) Release of fluid remains or of the over-aspirated volume from PJ1 back into the reservoir flask C: “Connect” pump to valves, “Open” valve1, activate button “Dispense“ as long as no liquid will be released any more or until the pump is out of range (“Plunger out of range“). In the case that there is still remaining liquid inside the tube, again aspirate some air using pump, and then dispense this volume via valve1: “Move” PJ1 to z-position 0, “Connect” pump to air, “Aspirate” (1-2x 30 µl), “Connect” pump to valves, “Move” PJ1 to z-position 28-30, “Dispense” (as long as all liquid is removed from the tube and pump reaches the initial position), “Close” valve1.
- (1) “Move” PJ1 to z-position 0.
- Trouble shooting: (3) when “aspirating“ about 30 µl, and then want to “Dispense“ just 5 µl, unfortunately not 5 µl reached the chip surface, but only 1.5 µl. When 3x “dispensing” 5 µl, 11.5 µl reached the chip surface... thus, dispensing an exact volume did not work well.....

IV. Manually performed spotting of 1µl of master mix onto reaction point B (PipeJet™2,PJ2)

- (1) “Move” LOC-slide to x-position 253.5 = flask C; “Move” PJ2 to z-position 33-37.
- (3) “Connect” pump to valves, “Open” valve2, “Close” valves 1 and 3; PSpeed = 20, PVol = 30 µl; press button “Aspirate“ (watch, if fluid is aspirated into the tube); pump stays connected to valve2.
- (1) “Move” PJ2 to z-position 0; “Move” LOC-slide to x-position 115 = reaction center B; “Move” PJ2 to z-position 30.
- (2) Adjust the following settings at PJ2: Istroke = 20 µm, vdown = 200 µm/ms, thold = 10 µs, vup = 2 µm/ms, n = 20, delay = 150 ms; validate settings with “Set PJ2“; start spotting with “Shoot PJ2“; about 1 µl will be spotted onto the chip surface.
- (3) After shooting, “Close” valve2 and “Connect” pump to air.
- (1) “Move” PJ2 to z-position 0.
- SAW-Control: 1 µl of fluid can now be move via SAW on the chip surface.
- (1) “Move” LOC-slide to x-position 253.5; “Move” PJ2 to z-position 28-30.
- (3) Release of fluid remains or of the over-aspirated volume from PJ2 back into the reservoir flask C: “Connect” pump to valves, “Open” valve2, activate button “Dispense“ as long as no liquid will be released any more or until the pump is out of range (“Plunger out of range“). In the case that there is still remaining liquid inside the tube, again aspirate some air using pump, and then dispense this volume via valve2: “Move” PJ2 to z-position 0, “Connect” pump to air, “Aspirate” (1-2x 30 µl), “Connect” pump to valves, “Move” PJ2 to z-position 28-30, “Dispense” (as long as all liquid is removed from the tube and pump reaches the initial position), “Close” valve2.
- (1) “Move” PJ2 to z-position 0.

V. Manually performed spotting of 5µl Sealing Solution onto reaction point C (PipeJet™3,PJ3)

- (1) “Move” LOC-slide to x-position 280 = flask A; “Move” PJ3 to z-position 33-37.
- (3) “Connect” pump to valves, “Open” valve3, “Close” valves 1 and 2; PSpeed = 20, PVol = 25 µl; press button “Aspirate“ (watch, if fluid is aspirated into the tube); pump stays connected to valve3.

- (1) “Move” PJ3 to z-position 0; “Move” LOC-slide to x-position 164 = reaction center C; “Move” PJ3 to z-position 28.
- (3) “Connect” valves to air = “Bypass“, thus, automatically a droplet is generated at PJ3 due to declined low-pressure, which drops automatically onto the chip surface due to gravity; as soon as the droplet reaches the surface, immediately “Connect” the pump to valves, to generate the low-pressure again and to stop the dispensing directly. OR: “Close” valve3, “Connect” valves to air (“Bypass“), “Open” valve3, wait for droplet touching the chip surface, “Close” valve3.
- (1) “Move” PJ3 to z-position 0.
- SAW-Control: 5 µl of Sealing Solution can now be moved via SAW on the chip.
- (1) “Move” LOC-slide to x-position 280; “Move” PJ3 to z-position 28-30.
- (3) Release of fluid remains or of the over-aspirated volume from PJ3 back into the reservoir flask A: “Connect” pump to valves, “Open” valve3, activate button “Dispense“ as long as no liquid will be released any more or until the pump is out of range (“Plunger out of range“). In the case that there is still remaining liquid inside the tube, again aspirate some air using pump, and then dispense this volume via valve3: “Move” PJ3 to z-position 0, “Connect” pump to air, “Aspirate” (1-2x 25 µl), “Connect” pump to valves, “Move” PJ3 to z-position 28-30, “Dispense” (as long as all liquid is removed from the tube and pump reaches the initial position), “Close” valve3.
- (1) “Move” PJ3 to z-position 0.
- Trouble shooting: (3) when “aspirating” about 25 µl, and then want to dispense 4x 5 µl, only 2.5 µl were dispensed... thus, dispensing did not work well with oil as well.
- Trouble shooting: (2) the “Shoot PJ3“-function did not work very well with oil... during “Shoot PJ3” single oil droplets were spread over the chip surface and did not combine to an increasing droplet volume. Furthermore, it was not possible to “shoot” more than about 2 µl – it seems that in that case the PJ-valve ran empty/out of liquid, as the stored fluid in the tube could not flow fast enough into the emptied PJ tip.... especially when valve was still connected to pump; but when connecting the valve to air, too much liquid was released and no shooting was possible.....
- (2) Additionally, parameter changes were tested, but did not succeed. Changes in “n“ = 20, 30, 40, 100 produced just a volume of maximum 2 µl when using “Shoot PJ3“. Changes in “delay“ of 100, 200, 500, 1000 and 2000 did not produce a larger dispensed volume than just 2 µl when using “Shoot PJ3“.

VI. To program automatic pipetting operations using the "(4) Batch mode"

- Write a working procedure first: step-by-step, what to do (example in **table 2**).
- Final instructions can then be typed into an Excel-sheet in the software according to the "Batch mode"-code (**figure 2**), or respectively, an already existing program can be loaded via "Open file" and then be changed (save changes via "Save file").
- Via "Batch execute" the list of orders will be run line-by-line.
- The generated working sheet of the „Batch mode" is saved with a .csv ending – this ending can simply be opened by Excel.
- Working sheets generated in Excel can easily be saved as .xls as well as .csv ending files, and thus can easily be opened in the "Batch mode".

Table 2. Step-by-step operating procedure of moving PJ1 to reaction center A and PJ3 to reaction center C, which can be transferred to an Excel file and translated according to the "Batch mode"-code.

	PJ1 = e.g. for master mix: use flask C (best centered) PJ3 = e.g. for Sealing Solution: use flask A (best centered)
	Axis Control settings:
(1)	"Xvel" / "Zvel" = 500 steps/s; press "Set Axis Speed" to validate new settings (Axis Control)
(1)	"Step width" = 0.01 mm (Axis Control)
	To "Aspirate" and "Dispense" the Sealing Solution/mineral oil (PipeJet™3):
(1)	1. "Move" LOC-slide to x = 280 mm to aspirate oil (Axis Movement)
(1)	2. "Move" PJ position to z = 33 mm (Axis Movement)
(3)	3. "Open" valve3 (V3) and keep the others closed (Valve Control)
(3)	4. "Connect" pump to valves by pressing "Valves" (Pump Control)
(3)	5. Set "PVol" to 25 µl (Pump Control)
(3)	6. Press "Aspirate" (Pump Control)
(1)	7. "Move" PJ to the initial position, z = 0 mm (Axis Movement)
(1)	8. "Move" LOC to x = 164 mm to dispense the oil in the reaction center C (Axis Movement)
(1)	9. "Move" PJ to z = 28 mm (Axis Movement)
(3)	10. To dispense the oil, "Connect" the valves to air by pressing "Bypass" (Pump Control)
(3)	11. Immediately after that the oil droplet reaches the chip surface, "Connect" the pump to valves by pressing "Valves" (Pump Control)
(1)	12. "Move" PJ to z = 0 mm (Axis Movement)
	To "Aspirate" and "Dispense" the master mix, H_2O, or else (PipeJet™1):
(1)	13. "Move" LOC-slide to x = 253.5 mm to pipette the master mix (Axis Movement)
(3)	14. "Open" valve1 (V1) and "close" valve3 (V3) (Valve Control)
(1)	15. "Move" PJ to z = 33 mm (Axis Movement)
(3)	16. Set "PVol" to 30 µl (Pump Control)
(3)	17. Press "Aspirate" (Pump Control)
(1)	18. "Move" PJ to z = 0 mm (Axis Movement)
(1)	19. "Move" LOC-slide to x = 62 mm to dispense the master mix in the reaction center A (Axis Movement)
(1)	20. "Move" PJ to z = 30 mm (Axis Movement)
(2)	21. Change PJ1 settings (PipeJet™ Control): Istroke(20µm); vdown(200µm/ms); thold(10µs); vup(2µm/ms); n(20); delay(100ms)
(2)	22. Press "Set PJ1" to validate the modifications (PipeJet™ Control)
(2)	23. Press "Shoot PJ1" (PipeJet™ Control)
(1)	24. "Move" PJ to z = 0 mm (Axis Movement)
(1)	25. "Move" LOC-slide to x = 300 for SAW control and PCR performance (Axis Movement)

Operating procedure written into the Excel working-sheet: "Batch mode"-code (**figure 2**)

To open a "Batch mode" file, press "Open file". To save setting changes in a file or to save a new created one, press "Save file". Saved files are stored in the folder "Methods" (drive C:\\Program Files/Biospot/Methods).

For typing any instructions into an Excel-sheet, the following settings have to be considered, providing the "Batch mode"-code:

A-D = (1) Axis Control and Axis Movement (target position of axis system)

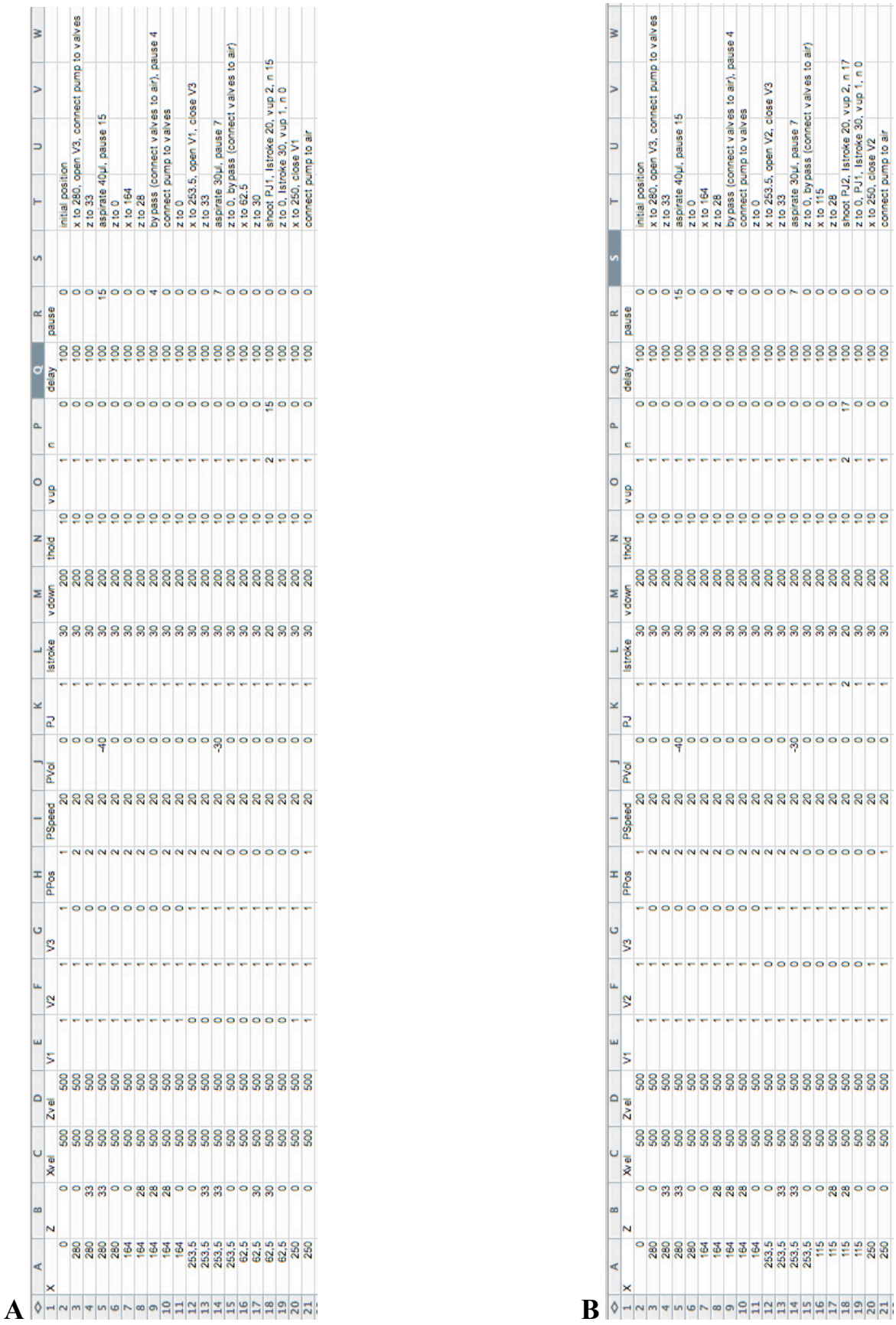
- X (A) and Z (B) = position of LOC-slide on x- and of PipeJets™ on z-axis
- Xvel (C) and Zvel (D) = speed of x-axis and z-axis (500 steps/s = standard)

E-J = (3) Valve Control and Pump Control (pumping behavior, valve position, valve velocity, volume)

- V1-3 (E,F,G) = valve 1-3 (1 = closed, 0 = open); keep all the valves closed until the aspiration or dispensing process is desired
- PPos (H) = connection of pump/air/valves (0 = bypass (valves/air), 1 = pump/air, 2 = pump/valves)
- PSpeed (I) = speed of aspiration/dispensing process (20 ms = standard)
- PVol (J) = volume of aspiration/dispensing in [µl]: (-) = aspiration, (+) = dispensing, 0 = standard (no pipetting activity)

K-R = (2) PipeJet™ Control (stroke, downstroke velocity, holdtime, upstroke velocity)

- PJ (K) = number of PJ, which is used to „shoot" (1, 2 or 3)
- Istroke (L) = penetration depth of piezo (5-36 µm, standard = 30 µm)
- Vdown (M) / vup (O) = down-/upstroke speed of piezo during dispensing process (downstroke 100-250 µm/ms (standard = 200), upstroke 1-10 µm/ms (standard = 1; in use = 2))
- thold (N) = adjustment of piezo holding time (10-1000 µs; standard = 10 µs)
- n (P) = number of repetitions of dispensing process (1-1000); 0 = standard = no "shooting" activity!!!
- delay (Q) = delay time between each repetition (1-2000 ms; standard = 100)
- Changes must be verified by pressing "Set PJ" button



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
1	X	Z	Xvel	Zvel	V1	V2	V3	PPos	PSpeed	PVol	PJ	Istroke	vdown	thold	vup	n	delay	pause		T	U	V	W
2		0	500	500	1	1	1	1	20	0	1	30	200	10	1	0	100	0		initial position			
3	280	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 280, open V3, connect pump to valves			
4	280	33	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 33			
5	280	33	500	500	1	1	1	0	20	-40	1	30	200	10	1	0	100	15		aspirate 40µl, pause 15			
6	280	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0			
7	164	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 164			
8	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 28			
9	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	4		bypass (connect valves to air), pause 4			
10	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		connect pump to valves			
11	164	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0			
12	253.5	0	500	500	0	1	1	2	20	0	1	30	200	10	1	0	100	0		x to 253.5, open V1, close V3			
13	253.5	33	500	500	0	1	1	2	20	0	1	30	200	10	1	0	100	0		z to 33			
14	253.5	33	500	500	0	1	1	2	20	-30	1	30	200	10	1	0	100	7		aspirate 30µl, pause 7			
15	253.5	0	500	500	0	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0, bypass (connect valves to air)			
16	62.5	0	500	500	0	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 62.5			
17	62.5	30	500	500	0	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 30			
18	62.5	30	500	500	0	1	1	0	20	0	1	20	200	10	2	15	100	0		shoot PJ1, Istroke 20, vup 2, n 15			
19	62.5	0	500	500	0	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0, Istroke 30, vup 1, n 0			
20	250	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 250, close V1			
21	250	0	500	500	1	1	1	1	20	0	1	30	200	10	1	0	100	0		connect pump to air			

A

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
1	X	Z	Xvel	Zvel	V1	V2	V3	PPos	PSpeed	PVol	PJ	Istroke	vdown	thold	vup	n	delay	pause		T	U	V	W
2		0	500	500	1	1	1	1	20	0	1	30	200	10	1	0	100	0		initial position			
3	280	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 280, open V3, connect pump to valves			
4	280	33	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 33			
5	280	33	500	500	1	1	1	0	20	-40	1	30	200	10	1	0	100	15		aspirate 40µl, pause 15			
6	280	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0			
7	164	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 164			
8	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 28			
9	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	4		bypass (connect valves to air), pause 4			
10	164	28	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		connect pump to valves			
11	164	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0			
12	253.5	0	500	500	0	1	1	2	20	0	1	30	200	10	1	0	100	0		x to 253.5, open V2, close V3			
13	253.5	33	500	500	0	1	1	2	20	0	1	30	200	10	1	0	100	0		z to 33			
14	253.5	33	500	500	0	1	1	2	20	-30	1	30	200	10	1	0	100	7		aspirate 30µl, pause 7			
15	253.5	0	500	500	0	1	1	0	20	0	1	30	200	10	1	0	100	0		z to 0, bypass (connect valves to air)			
16	115	0	500	500	1	0	1	0	20	0	1	30	200	10	1	0	100	0		x to 115			
17	115	28	500	500	1	0	1	0	20	0	1	30	200	10	1	0	100	0		z to 28			
18	115	28	500	500	1	0	1	0	20	0	2	20	200	10	2	17	100	0		shoot PJ2, Istroke 20, vup 2, n 17			
19	115	0	500	500	1	0	1	0	20	0	1	30	200	10	1	0	100	0		z to 0, PJ1, Istroke 30, vup 1, n 0			
20	250	0	500	500	1	1	1	0	20	0	1	30	200	10	1	0	100	0		x to 250, close V2			
21	250	0	500	500	1	1	1	1	20	0	1	30	200	10	1	0	100	0		connect pump to air			

B

Figure 2. Programming operating procedures in "Batch Mode". Operating procedures can be written into an Excel working-sheet. Parameters can be typed into the columns according to the desired operation to be performed. A+B) Operating procedures for pipetting 1 µl of fluid to reaction point A (A) or B (B) and dispensing 5 µl of Sealing Solution to reaction center C, expressed according to the "Batch mode"-code.

9.1.6 LOP of Fluorescence Reader

"Fluorescence detection for applications like RT-PCR or microarray on the LOC system"

A. Material

- BioSpot® device and software "BioSpot®" (BioFluidix GmbH, Freiburg, Germany)
- CytoCycler (PCR device incl. chip-holder, temperature control device, SAW control high frequency (HF) generator, particular software "CytoCycler" (Advalytix AG/Beckman Coulter Biomedical GmbH, Munich, Germany))
- Electronics (electronic control): LED power control box (self made), trigger signal break-out box (NI SCB-68 with the PCI ADC/DAC capture the trigger signal, activates the image capture and image processing process; Quick Reference Label, S-Series Devices, National Instruments Germany GmbH, Munich, Germany)
- Optics: - black & white CCD camera (Rolera-XR, QImaging, Surrey BC, Canada)
  - Filter sets (Interferenzfilter of BrightLine series, AHF Analysentechnik AG, Tübingen, Germany): excitation filter λ_{max} = 498 nm (spread 35 nm = 464 500 nm); emission filter λ_{max} = 536 nm (spread 40 nm = 516-556 nm); both)
  - light source: blue LED (λ_{max} = 470±2 nm LUXEON Rebel LXML-PB01 0023, 3.4 V forward bias, 0.7 A operating current)
- Software: "QCapture" or "QCapture PRO 6.0" (QImaging, Surrey BC, Canada), "LED_Switch.VI", "Norbert.VI", "Grand_NIVision_Intensity_Consec_Subtract_ Loopback_NewCamera.VI" (LabVIEW 8.6, National Instruments Germany GmbH, Munich, Germany)

B. Operating procedure

I. Start BioSpot® (for moving LOC chip-holder to CCD camera (Fluorescence Reader))

- Prepare a test chip into the LOC chip-holder with a droplet in place (1 µl of water covered with 5 µl of mineral oil (Sealing Solution) at reaction center B) (see detailed description of chip installation in the "LOP of LOC chips", **chapter 9.1.3**).

- Switch on BioSpot® control device, start software "BioSpot®" (desktop; see "LOP of BioSpot®", **chapter 9.1.5**).
- Move CytoCycler (LOC chip-holder slide) to CCD camera (x-axis position = 460).
- Leave "BioSpot®" software open, stored in the background.

II. Run "QCapture" (optimize chip position and camera settings)

- Transfer CCD camera from the microscope and fix it to the holder (big golden screw).
- Switch on CCD camera and connect to computer via firewire connection.
- Start software "QCapture" (desktop; **figure 1**).
- Click "Acquire" for activating window "Live Preview" (the software provides a live image of the chip surface).
- Center camera image to the test droplet, optimize settings like magnification as well as focus (the image from the camera has to be focused sharply).
- OR start software "QCapture Pro 6.0" (desktop; **figure 2**); click on the camera symbol for activating a settings box, where by clicking the "Preview" button a live preview is provided.

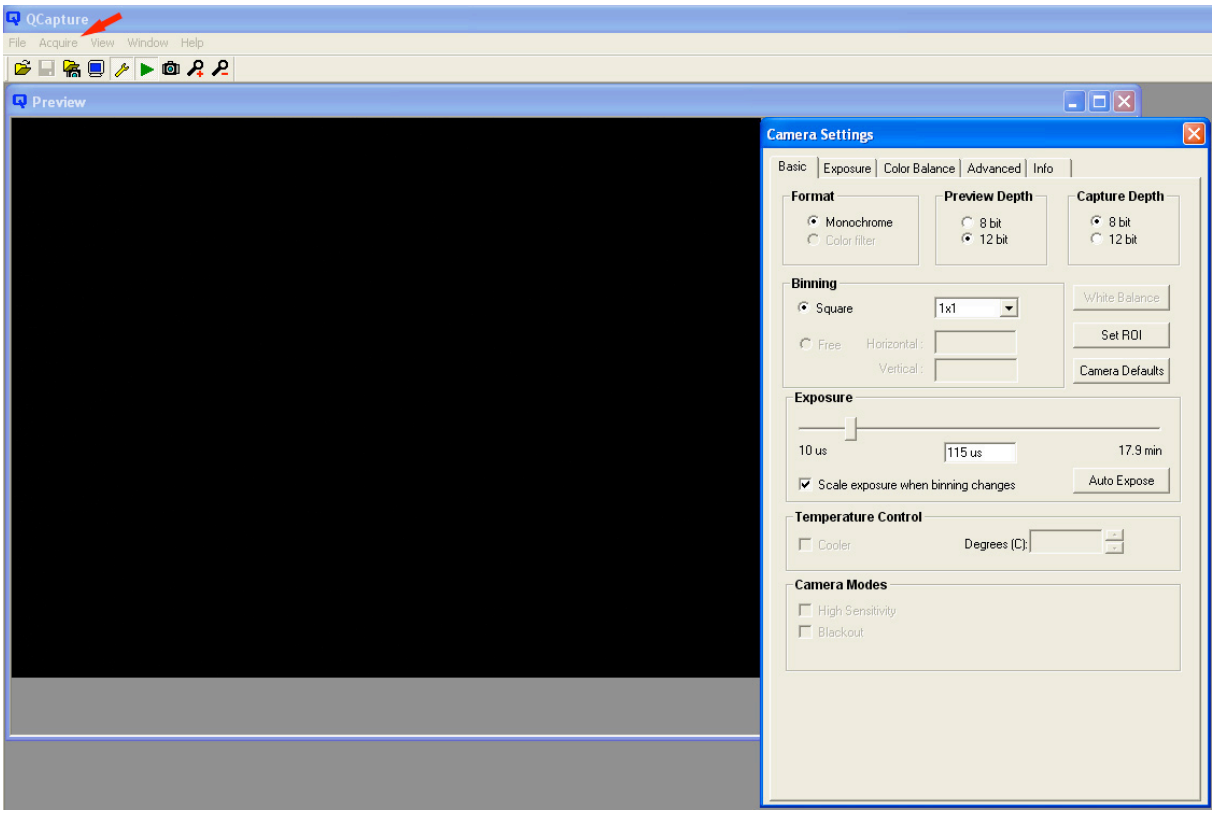


Figure 1. Screenshot of software "QCapture". Via activating the button "Acquire" (red arrow) a window opens providing a live preview image.

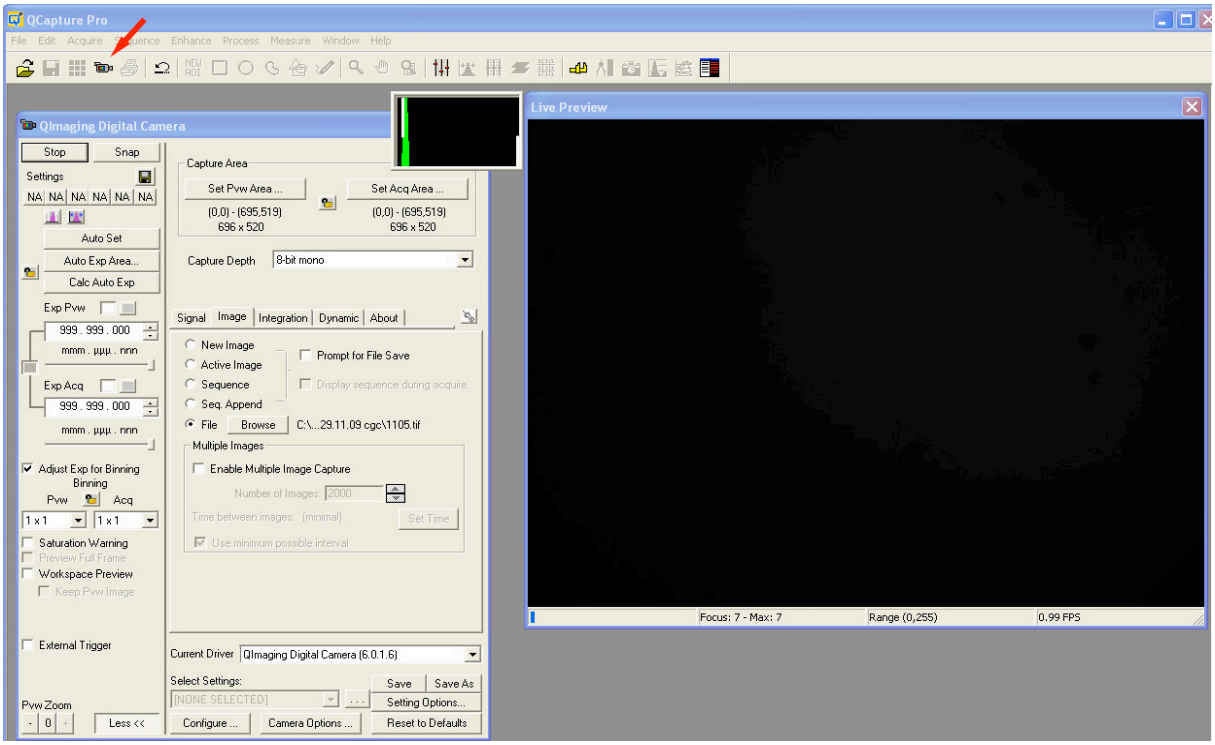


Figure 2. Screenshot of software "QCapture Pro 6.0". Via activating the "Camera" symbol (red arrow), a window opens providing settings for the live preview. Operating the button "Preview" in these settings window provides then the live image of the camera.

III. Run "LED_Switch" (check LED state)

- Switch on LED power control box (red button at the grey self-made box).
- Start software "LED_Switch" (desktop; **figure 3**).
- Run program by clicking the white arrow symbol; activate the center switch for turning blue LED light on and off; adjust LED illumination (the focused light must be centered onto the middle of the test droplet); turn LED off.

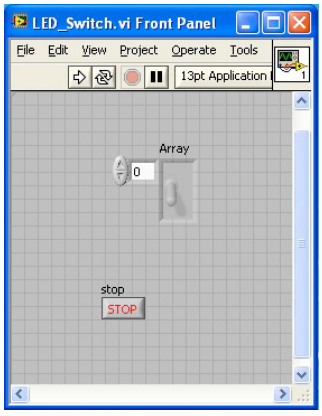


Figure 3. Screenshot of software "LED_Switch.VI". The white arrow button, the red-circle button and the centered switch are needed to operate the LED light illumination.

- Stop program by clicking the red-circle button; quit software “LED_Switch” as well as the “QCapture” software.

Important: after these preliminary steps the “QCapture” software as well as the “LED_Switch” software need to be stopped, as only one procedure at a time can have access to the camera as well as to the LED light source (so either “QCapture+LED_Switch” OR “Norbert.VI/Grand_NIVision...” can be run).
Thereafter, the test chip can be removed out of the cavity of the CytoCycler device and a new chip has to be fixed in place, whereon the PCR shall take place.

IV. Run LabVIEW program

- Switch on CCD camera and connect to computer via firewire connection.
- Switch on LED power control box (red button at the grey self-made box).

During PCR, images taken via the LabVIEW programs are stored in folder D://pic (file name = OriginalXX.tiff / OriginalXX.jpeg) → after the PCR these images need to be stored in a separate folder, otherwise images will be overwritten by new saved images when image taking programs are re-opened and run again. The software “Grand_NIVision...” also generates images named ProcessedXX.tiff / ProcessedXX.jpeg – these pictures represent subtracted images showing the calculated difference in fluorescence intensity when subtracting the previous image from the actual image. During PCR, values of fluorescence intensity are stored continuously in the domain named “Array”; additionally, at the “Grand_NIVision...” software, an excel file is generated storing these values when the program is stopped.

Assure that the trigger signal from the PCR CytoCycler is fed into the trigger signal break-out box which itself must be connected to the interface board placed in a computer slot. From the break-out box another two lines have to be connected to the LED power control box. They provide the trigger signal for the LED to be switched on and off. The LED power control box must be switched on as well (red button).

Run “Norbert.VI” (for manual image taking)

- Start software “Norbert.VI” (desktop; **figure 4**).
- Set exposure time (in milliseconds) to desired values like e.g. 100, 200, 400, 1000, and so on in the box “Exposure” (**figure 4**, red arrow 1).
- Run program by clicking the white arrow symbol (**figure 4**, red arrow 2); now images can be taken manually at any time point by clicking the button “Take picture” (**figure 4**, red arrow 3).
- Run PCR (see issue V “Start CytoCycler”).
- Stop program by clicking the red-circle button (**figure 4**, red arrow 4); quit software “Norbert.VI”.
- Save images stored in folder D://pic to a separate folder, as otherwise they will be overwritten.

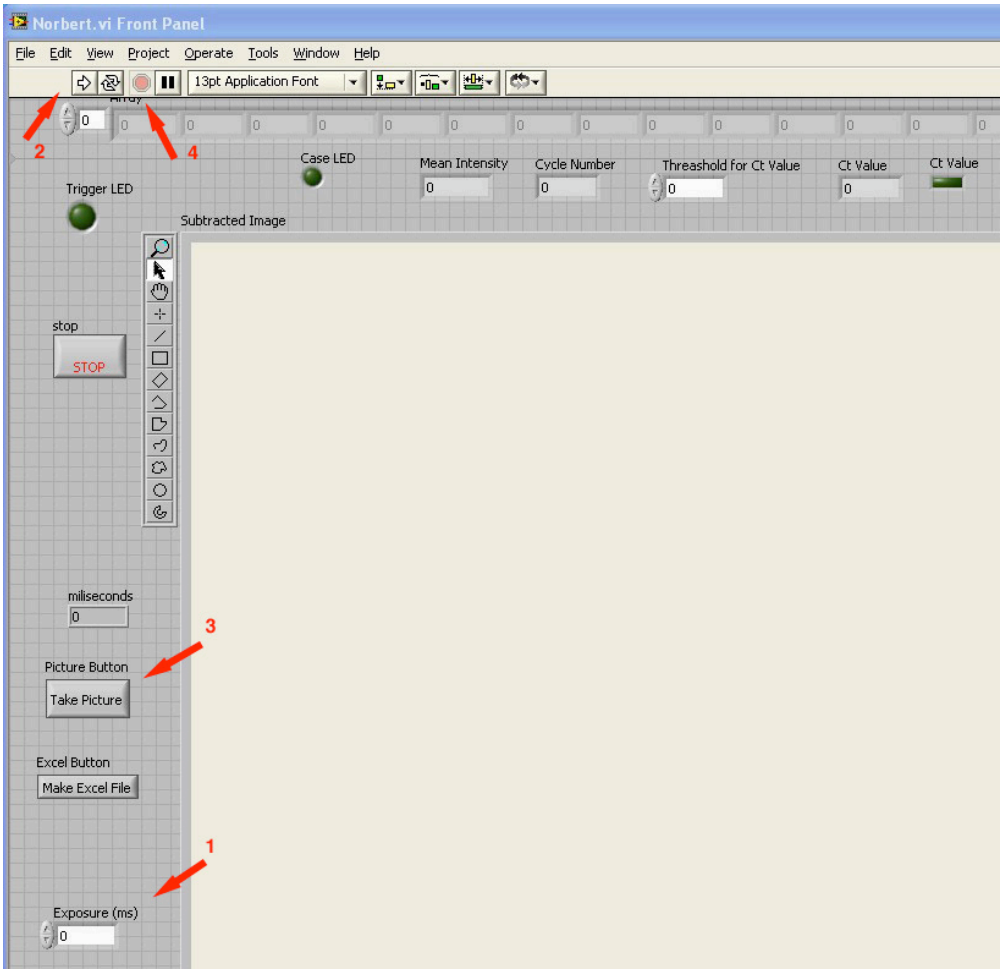


Figure 4. Screenshot of software “Norbert.VI” for taking pictures manually. The white arrow button, the red-circle button and the “Take Picture” button are needed to operate the software, while exposure time needs to be set as well. Measured fluorescence intensities of taken images are shown in the box “Mean Intensity” and are stored consecutively in domains arranged in an array-like manner below the starting button.

or “Grand_NIVision_Intensity_Consec_Subtract_Loopback_NewCamera.VI” (for automatic image taking)

- Start “Grand_NIVision_Intensity_Consec_Subtract_Loopback_NewCamera.VI” (desktop; **figure 5**).
- Set exposure time (in milliseconds) to desired values like e.g. 100, 200, 400, 1000, and so on in the box “Exposure” (**figure 5**, red arrow 1).
- Run program by clicking the white arrow symbol (**figure 5**, red arrow 2); now images are taken automatically at distinct time points; time points depend on the position (front view: left = 1.step of PCR cycle, middle = 2.step, right = 3.step), where the trigger signal break-out box is connected to the backside of the temperature control box for performing 2-step or 3-step PCR: plug 2/middle = images taken at the end of the second temperature step during PCR (around 60°C), plug 3/right = images taken at the end of the third temperature step during PCR (around 72°C)).
- Run PCR (see issue V “Start CytoCycler”); the program now waits for the trigger signals from the PCR CytoCycler.

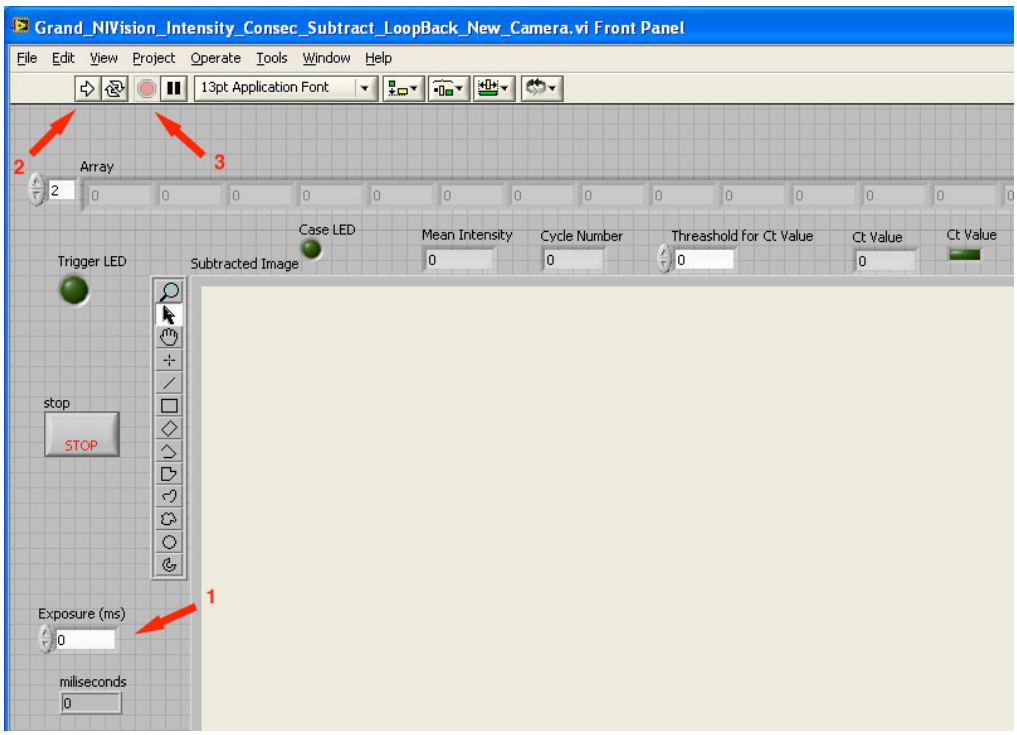


Figure 5. Screenshot of “Grand_NIVision_Intensity_Consec_Subtract_Loopback_NewCamera.VI” software for taking pictures automatically. The white arrow button and the red-circle button are needed to operate the software, while exposure time needs to be set as well. Measured fluorescence intensities of taken images are shown in the box “Mean Intensity” and are stored consecutively in domains arranged in an array-like manner below the starting button.

- Stop program by clicking the red-circle button (**figure 5**, red arrow 3); quit software "Grand_NIVision...".
- After the PCR, automatically an Excel file is generated showing a summary of measured values of fluorescence intensity.
- Save images stored in folder D://pic to a separate folder, as otherwise they will be overwritten.

V. Start "CytoCycler" (performing PCR)

- Install new chip in LOC chip-holder device; switch on temperature control box, start software "CytoCycler" (desktop) and choose PCR program/settings (see "LOP of LOC chips", **chapter 9.1.3** and "LOP of LV-PCR", **chapter 9.1.4**).
- Pre-heat each chip for about 15 min to 95°C (during this procedure, the material of the chip expands due to the high temperature – this prevents the droplet afterwards, when starting the initial denaturation step of the PCR, to be moved/deformed due to material stress).
- Run PCR using 1 µl of master mix, covered by 5 µl of Sealing Solution (see "LOP of LV-PCR", **chapter 9.1.4**).