Isolation and multiplex genotyping for circulating tumor cells

Magnetic Isolation and moleculaR Analysis of single CircuLating and disseminated tumor cElls on chip

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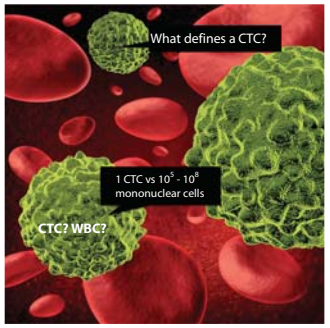
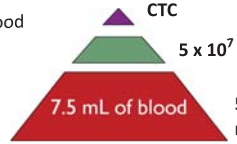
Circulating tumor cell (CTC): what and why?



Tumor cells shed from the primary tumor to blood and seed new tumors at other organs (metastasis, cause of 90% cancer deaths).



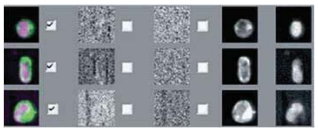
The **counting** (most important measure[1]) and **multi-gene analysis** (useful for personalized treatment[2]) of **individual CTCs** are important for cancer prognosis.

CTC properties, e.g. size, are often similar to some normal cells (typically lymphocytes); CTCs also very heterogeneous from each other (e.g. size and surface marker expression).

Why MIRACLE, and how?

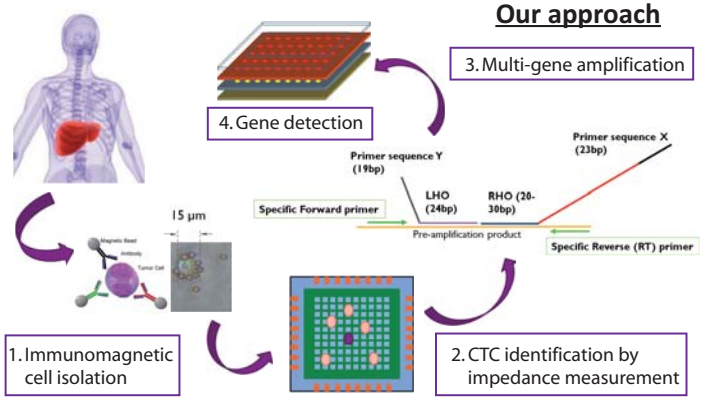
State of the art

- Single-marker based magnetic cell isolatin
- Cell viability not preserved, incompatible with downstream DNA/RNA analysis
- No possible for individual cell analysis
- No gene analysis

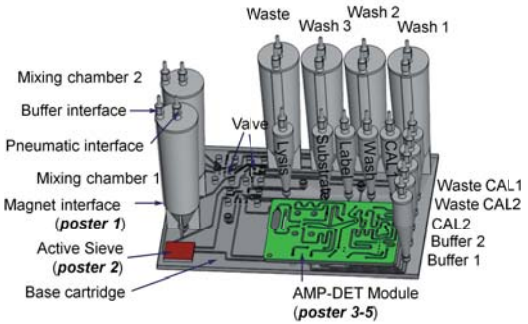


Fluorescent CTC images are presented in a gaellery format for confirmation

Our approach



Inside the MIRACLE box



Design specifications

- **7.5 mL** patient peripheral blood
- Detection limit: **1 CTC** per sample
- Specificity & sensitivity: **> 90%**
- Marker for 9mmunomagnetic isolation: **EpCAM & EMT**
- CTC distinguishment from WBCs: non-invasive **electrical impedance spectroscopy**
- MLPA gene number: **7** (core breast cancer panel) **& 16** (prostate cancer panel)
- MLPA sensitivity: **single CTC**
- MLPA detection limit: amplicons from **single CTC**

Reference: [1]EU FP-7 MIRACLE project (http://www.miracle-fp7.eu/) / [2]Stakenborg et al, Automated genotyping of circulating tumor cells, Exp. Rev. Mol. Diagn., 2010.

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MIRACLE

Magnetic Isolation and moleculaR Analysis of single CircuLating and disseminated tumor cElls on chip

CTC Isolation and Molecular Characterization in one Device

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Immunomagnetic cell isolation

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For the isolation of the CTCs an immunomagnetic approach was chosen. As particles 300nm beads are used due to the compatibility of their size to the following sieving step of the MIRACLE project. The functionalization of the beads with EpCam was developed and executed by Adnagen. One of the mayor requirements for the MIRACLE project was to design the separation device as a disposable with low fabrication costs and compatible to mass fabrication techniques. Therefore a "passive mixing" approach was identified. This mixing concept bases on the effect of exchanging inner and outer parts of the liquid when it gets pumped through a channel with different square sections.
Fig.1 shows the isolation prototype.

Fig.1: The Isolation and counting prototype

The isolation reservoir is connected via a channel with the mixing reservoir. By alternating transfer of the sample / magnetic beads solution between the two reservoirs, the magnetic beads are mixing inside the vessels. After the incubation for binding the CTCs to the magnetic beads, a permanent magnet is placed on the sidewall of the isolation reservoir to immobilize the cell/beads complexes on the sidewall (). The sample liquid (supernatant without magnetic beads) is transferred via the turning valve to the backside of the device and is stored inside the waste reservoir. In a next step the beads are washed from the sidewall by the washing buffer and the mixing and immobilization process is repeated. After 3 washing step, the cell / beads complexes are resuspended by a transfer buffer and transferred from the prototype to the next microfluidic module.

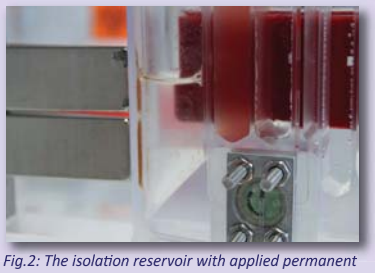

Fig.2: The isolation reservoir with applied permanent magnets

Cell line	Passage	System	No. of spiked cells	No. of isolated cells	Isolation efficiency
MCF-7 IMM	16	Standard	21	15	71%
MCF-7 IMM	16	MIRACLE	20	16	80%
MCF-7 IMM	17	Standard	20	16	80%
MCF-7 IMM	17	MIRACLE	20	12	60%
MCF-7 IMM	17	MIRACLE	20	13	65%
MCF-7 IMM	17	Standard	20	16	80%
MCF-7 IMM	17	MIRACLE	21	17	81%
		Standard	77%		
		MIRACLE	71%		

Tab.1: isolation efficiency of the MIRACLE isolation prototype with the laboratory standard.

To test the functionality of the prototype, 20 MCF-7 cells were labeled with a fluorescent dye and spiked into cell medium. The isolation was performed in the MIRACLE isolation prototype as well as in the laboratory standard protocol following the same processing steps. Afterwards the number of recovered cells in the final suspension were counted using a microscope. The results presented in Tab.1 shows that the isolation efficiency is comparable to the isolation standard and therewith a suitable isolation approach for the MIRACLE project.

Daniel Latta, Ralf Himmelreich - Fraunhofer ICT-IMM

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Prototype amplification and detection module

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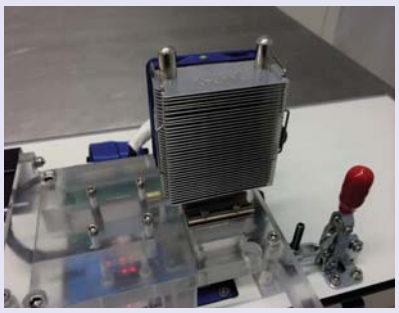
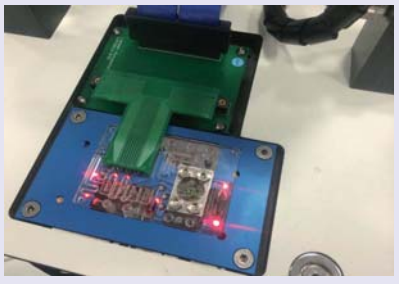
The current Miracle system comprises of three modules.



1) Thermal Cycler

This is a prototype module used to perform polymerase chain reaction (PCR) on a DNA sample inside a disposable micro fluidic chip. The core function of this module is to set up and hold any temperature within the range of 10°C to 120°C with a temperature accuracy of 1°C. This functionality is achieved using a peltier Thermo-Electric Controller (TEC). The TEC is controlled by a Labman developed software algorithm which uses the principle of PID control to heat or cool micro litres of fluid within a PCR chamber in the fluidic chip. Precise temperature accuracy is maintained using a PID controller which adjusts a PWM signal controlling the TEC. Labman software allows a user to set up temperature profiles which can individually specify a temperature and time duration for each specific part of the PCR cycle. This gives the module a reconfigurability making it applicable for any of the multitude of PCR cycling profiles developed. The software also plots the temperature data in real time providing the user a feedback of the currently active temperature profile and performance characteristics of the thermal cycling process.



2) DNA Detection

The second module of the Miracle system is a DNA detection potentiostat. 64 individual gold electrode chips are fabricated and functionalised with complimentary bio-marker DNA at different concentration levels by Rovira i Virgili University in Spain.
A Labman developed potentiostat measures electron flow through each of these individual electrodes in parallel (current levels on each electrode is of the order of nano Amperes). The parallel current measurement technique developed and implemented in this module makes it a very fast potentiostat compared to its commercial counterparts. Current measurement data from each of the electrodes is presented to the user in tabular and graph formats. Plans are under-way to expand this system to 256 electrodes with simultaneous parallel current measurement and cyclic voltammetry capabilities.

3) DNA Fluidic control

This is the third module comprising of control electronics, valves and syringes needed to control the flow of micro litres of fluid inside the micro-fluidic chip. Fibre optic light sensors and pressure sensors are used to precisely locate the liquid inside the chip providing automatic control of syringe drives to perform the required flow.

Joe Roberts, Labman Automation Ltd

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Data Analysis

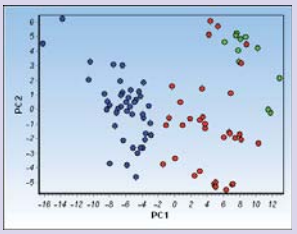
GenEx - The ultimate tool for analyzing qPCR data

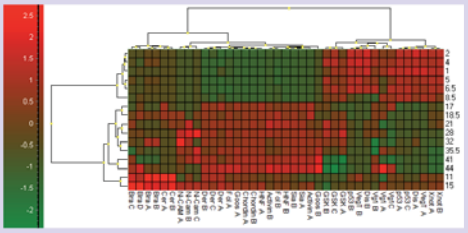
Among the new features you find comprehensive CLSI compliant analysis, which satisfies the requirements of regulatory bodies, including:

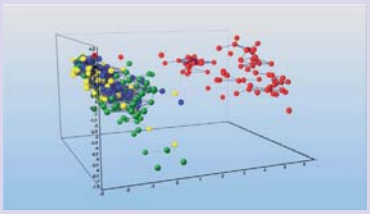
- Estimating PCR efficiency from standard curve, including confidence interval
- Testing for outliers in a qPCR standard curve
- Testing for linear model in a qPCR standard curve
- Testing for dynamic range in a qPCR standard curve
- Estimating random error of a qPCR standard curve
- Estimating limit of detection including confidence interval of a qPCR assay/test
- Estimating limit of quantification of a qPCR assay/test
- Estimating concentrations including confidence intervals of unknowns based on qPCR standard curve
- Evaluation of Precision of a qPCR assay/test
- Verification of precision of a qPCR assay/test

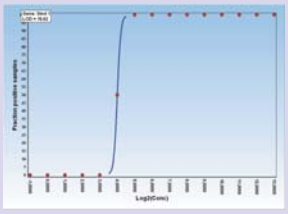
Other New Analysis Features include:

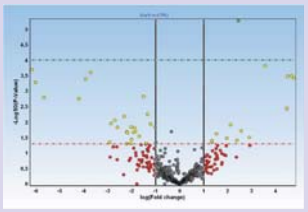
- Histogram plot for single cell analysis
- Repeated Measures ANOVA
- Survival Analysis
- Receiver Operator Characteristics (ROC)

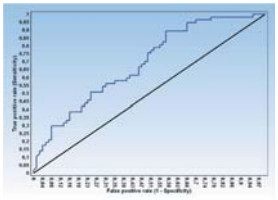


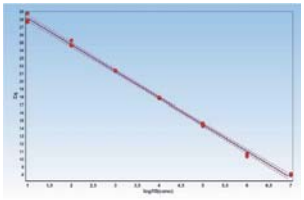


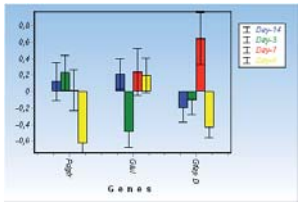


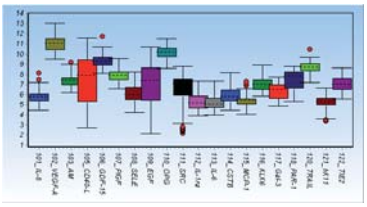


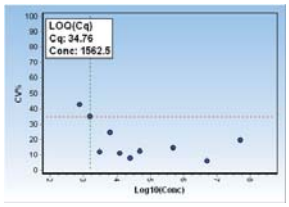












Amin Forootan, MultiD

MIRACLE

Magnetic Isolation and molecular Analysis of single Circulating and disseminated tumor cElls on chip (01.09.10-31.08.14)

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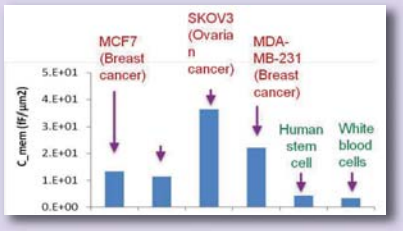
Single tumor cell characterization by an electrical impedance spectroscopy array

CTC identification from WBC by its unique electrical impedance

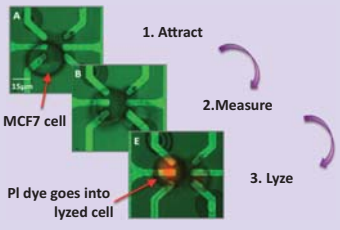
Motivation

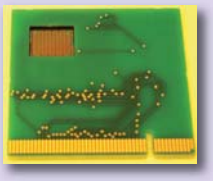
- CTCs are always together with high number (e.g. 2,000) of WBCs after any cell pre-enrichment method (e.g. immunomagnetic isolation)
- A method is needed to identify and isolate CTCs from WBC background, ideally with **preserved cell viability** (not immune-cytochemical staining)



Our approach

- Tumor cells exhibit higher **electrical membrane capacitance** than normal cells
- We measure the cell membrane capacitance on a micro electrode array Active Sieve by:
  Step 1: Attracting cells to the electrodes by dielectrophoretic force
  Step 2: Measuring the cell membrane impedance
  Step 3: Cell transfer (e.g. for culture) or **electrical cell lysis** to release DNA/RNA after CTC identification

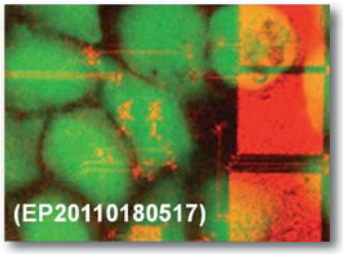


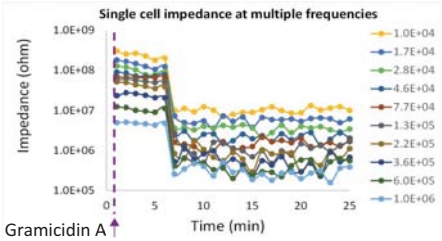


Advantages:

Fast, high throughput measurement
(10,000 cell measurement on a transistor chip)
Cells are viable after measurement
Individually addressable CTC harvest / lysis

A cellular assay platform with single cell monitoring capability





Add Gramicidin A

- Cell activity in response to treatments (e.g. drugs) are monitored by electrical impedance spectroscopy (e.g. impedance drop at cell death)
- 10,000 parallel cell measurement with **single cell resolution**

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Magnetic Isolation and molecular Analysis of single Circulating and disseminated tumor cElls on chip (01.09.10-31.08.14)

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MLPA on and off chip, single cell sensitivity

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RT-MLPA approach

The MLPA (multiplexed ligation-dependent probe amplification) procedure (Schouten et al.) has been developed and optimized to allow detection of transcripts from single cells and is intended for molecular analysis of circulating tumor cells (CTCs) in the MIRACLE device but could also prove useful in other applications where the expression profiles of a panel of transcripts in single cells are of interest. (Figure 1)

For both of the gene panels used in MIRACLE, gene specific RT primers have been developed, as well as two sets of probes. One set is labeled with stuffer regions of varying length, for detection using gel or capillary electrophoresis. The other set is labeled witha molecular barcode, for detection mediated by hybridization on an array.

Figure 1 – Overview of the RT-MLPA procedure. mRNA is converted into cDNA and amplified using gene specific primer. MLPA probes are allowed to hybridize to the amplified targets,and if the oligonucleotides of the probes hybridize adjacent to each other they can be ligated and amplified using a universal MLPA primer pair.

Off chip

When analyzing CTCs, single cell sensitivity and specificity are necessary: residual blood cells from the isolation step are unavoidable and must be taken into account for MLPA since mRNA molecules will be amplified regardless of their cell of origin. We have good indication that our protocol can handle contamination from blood cells and still gives a specific cancer cell signal (Figure 2).

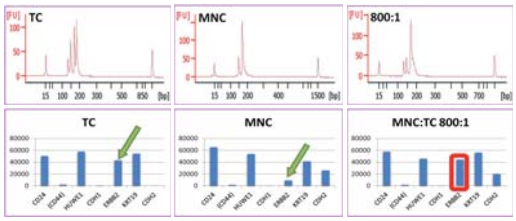


Figure 2 – Acceptable blood cell contamination levels in the RT-MLPA procedure. Extracted total RNA from TCs and MNCs has been mixed in different ratios, well as tested individually, and at a ratio of 800:1 (corresponding to 160 ng MNC RNA spiked with 200 pg TC RNA). One peak can be detected which is only visible in the TC positive control but not the MNC positive control

An example of our optimization efforts is the combination or separation of the ligation and amplification steps in MLPA procedure. When combined, all three samples show a KRT19 peak (Figure 3) which is not expected to yield positive signals in MNC samples. However, upon separating these two steps, the product is now amplified in a cell specific manner (Figure 3). Furthermore, our protocol demonstrates single cell sensitivity (data not shown).

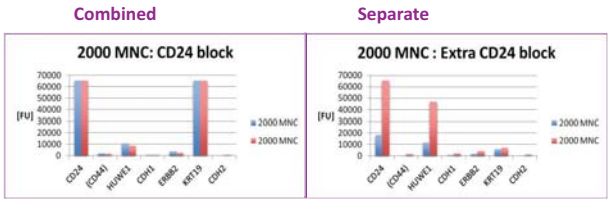
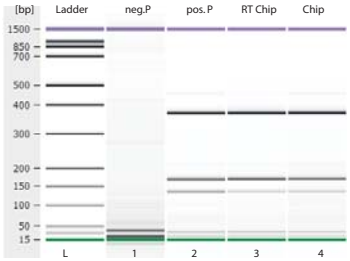


Figure 3 – The effect of combined or separate ligation and amplification of the MLPA probes. When combining the two steps a positive signal in pure MNC samples is seen for the epithelial marker KRT19. This false signal is lost when the reagents for the two steps are added separately and the ligase is inactivated after the ligation step.

On chip

The method was tested on the injection molded prototypes. 20 MCF-7 cells were spiked into lysis buffer and amplification was performed in an amplification and detection prototype and in a bench top cycler. Figure 6 shows gel electrophoresis picture for four different cases. It can be seen that for a low number of cells the amplification and detection prototype performs with an efficiency comparable to the bench top cycler.



Elin Johanson, Linda Kvastad - KTH, Karolinska Institutet Science Park

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Multi-gene detection chip

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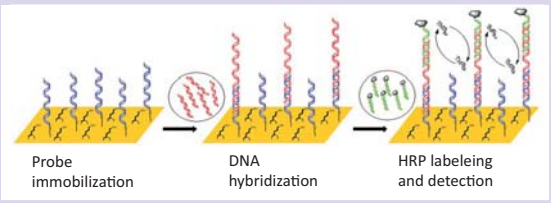
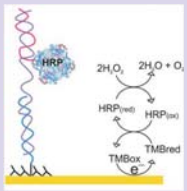
Electrochemical assay

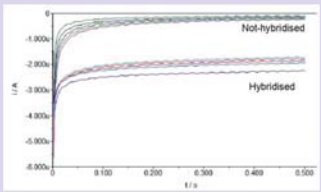
Detection of surface bound DNA duplexes using a secondary DNA probe coupled to the HRP enzyme which oxidizes TMB and which is further reduced at the electrode surface and therefore quantified using fast electrochemical pulse amperometry.



Multiplex gene detection

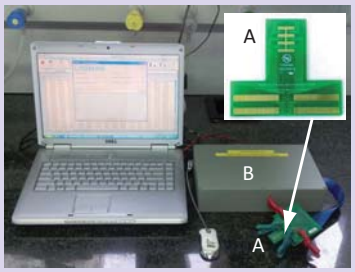


Assay schematic



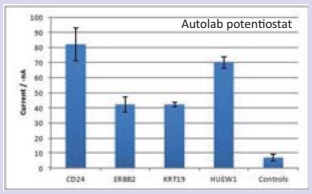
Electrochemical pulse amperometry

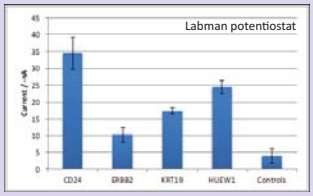
Labman's multichannel potentiostat



Experimental set-up.
A) DNA chip,
B) Labman's potentiostat

- Multichannel potentiostat is capable to perform simultaneous measurement of the 64 electrodes
- Read-out in less than 5 seconds.
- Results on Labman potentiostat (64 simultaneous channels) equivalent to those obtained on commercial Autolab (64 sequential channels)





Electrochemical analysis of single cell single stranded MLPA for 4 individual markers extracted and amplified from single tumor cells

Electrode chip

Disposable PCB- based 64-electrode DNA array

- 64 individually addressable electrodes.
- Printed circuit board (PCB) technology.
- Highly flexible multi-gene detection chip.
- Disposable chips.
- Stable and functional for >110 days.
- High chip-to-chip reproducibility.

- Developed an electrochemical platform for the detection of genes involved in breast cancer using i) low-cost electrode microarrays manufactured on standard printed circuit board (PCB) substrates and ii) multichannel potentiostat with single tumor cell sensitivity.
- Electrochemical DNA microarrays offer high sensitivity, lower cost and ease of implementation in highly integrated microsystems.

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