Tracking the trace: Elucidating the source of circulating tumor cells and circulating tumor DNA

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Background and aim

A promising possibility to obtain molecular characteristics of a tumor in real-time is the analysis of liquid biopsies including circulating tumor cells (CTCs) and cell-free circulating tumor DNA (ctDNA). Both analytes can be sampled minimal invasively and provide highly relevant biomarkers for diagnostic approaches in the field of precision medicine. Although it is widely accepted that CTCs are the seeds of metastatic disease, it is not well understood which factors contribute to their release into blood. Similar holds true for the release of ctDNA, as multiple mechanisms such as apoptosis, inflammation, proliferation or vascularization are described, but partly contradicting each other [1-4].

There is an urgent need to shed light on how tumor cells and tumor DNA can escape tumor sites and enter blood circulation. We will use next generation sequencing (NGS) analysis on liquid biopsies and corresponding tumor tissue samples of patients to identify mutations present in CTCs and ctDNA. Using *in situ* sequencing technology (ISS) we will map the mutations detected in CTCs and ctDNA back to the tumor to identify their originating area. Additional ISS panels for immunophenotyping, pathways and other cell types will allow in depth characterization of the respective tumor areas.

In situ sequencing (ISS) technology

ISS technology is used to detect multiple mRNA transcripts (>200 targets) in parallel with single base resolution directly on tissue sections by using just five different fluorescent dyes. First, mRNA transcripts are reverse transcribed to cDNA and specific padlock probes (PLPs) bind cDNA with juxtaposed ends next to each other which allow the ligation of the PLPs. The resulting circularized PLPs are enzymatically multiplied by rolling circle amplification (RCA). Each PLP contains a unique ID sequence, which is associated to a combinatorial barcode scheme (e.g. 1242, Fig. 1A) that can be decoded sequentially by several cycles of hybridizations with bridge probes and readout detection probes. The analysis of the data is performed in CellProfiler and MATLAB [5].

Preliminary data and results

- A panel of 260 genes has been designed including markers for apoptosis, autophagy, necrosis, proliferation, vascularization, oxidative stress, differentiation, invasion, cell phenotyping and immune cell composition.
- Optimization of the filter set to reduce the crosstalk of channels.
- Optimization of the ISS library preparation based on *Malat1* expression on colorectal cancer (CRC) tissue (Fig. 2 and Tab.1).

Malat1	Signals/area (1*10^6 pixel)	Signal/background ratio	Signal/noise ratio
pepsin 30min	2327	2,51	7,22
pepsin 10min	1317	3,87	4,90
citrate buffer + microwave - 45min	1294	3,41	5,21
citrate buffer + steamer - 45min	**3858**	**3,89**	**7,02**

Tab. 1: Optimization of the library preparation
The permeabilization step on CRC tissue was performed with four different conditions: pepsin treatment for 30 and 10 minutes and citrate buffer treatment in a microwave and a steamer for 45 minutes. The citrate buffer treatment in combination with a steamer for 45 minutes provided the best results regarding the 3 calculated values signals/area, signal/background ratio and signal/noise ratio.

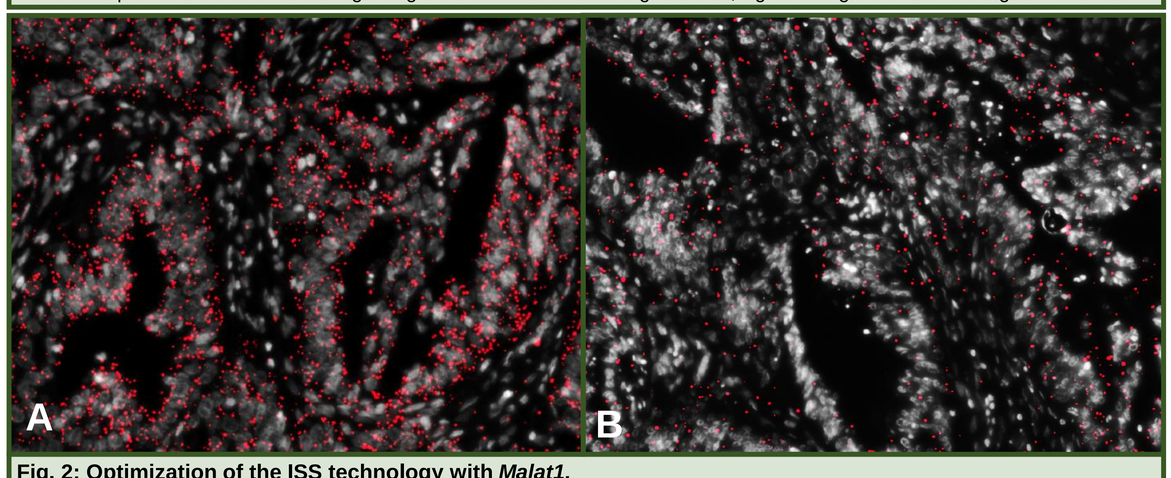

Fig. 2: Optimization of the ISS technology with *Malat1.*
In situ sequencing was performed on CRC tissue with different permeabilization conditions to achieve the optimal results (A). (A) Tissue was treated with citrate buffer in a steamer for 45 minutes. (B) Tissue was treated with pepsin for 10 minutes.

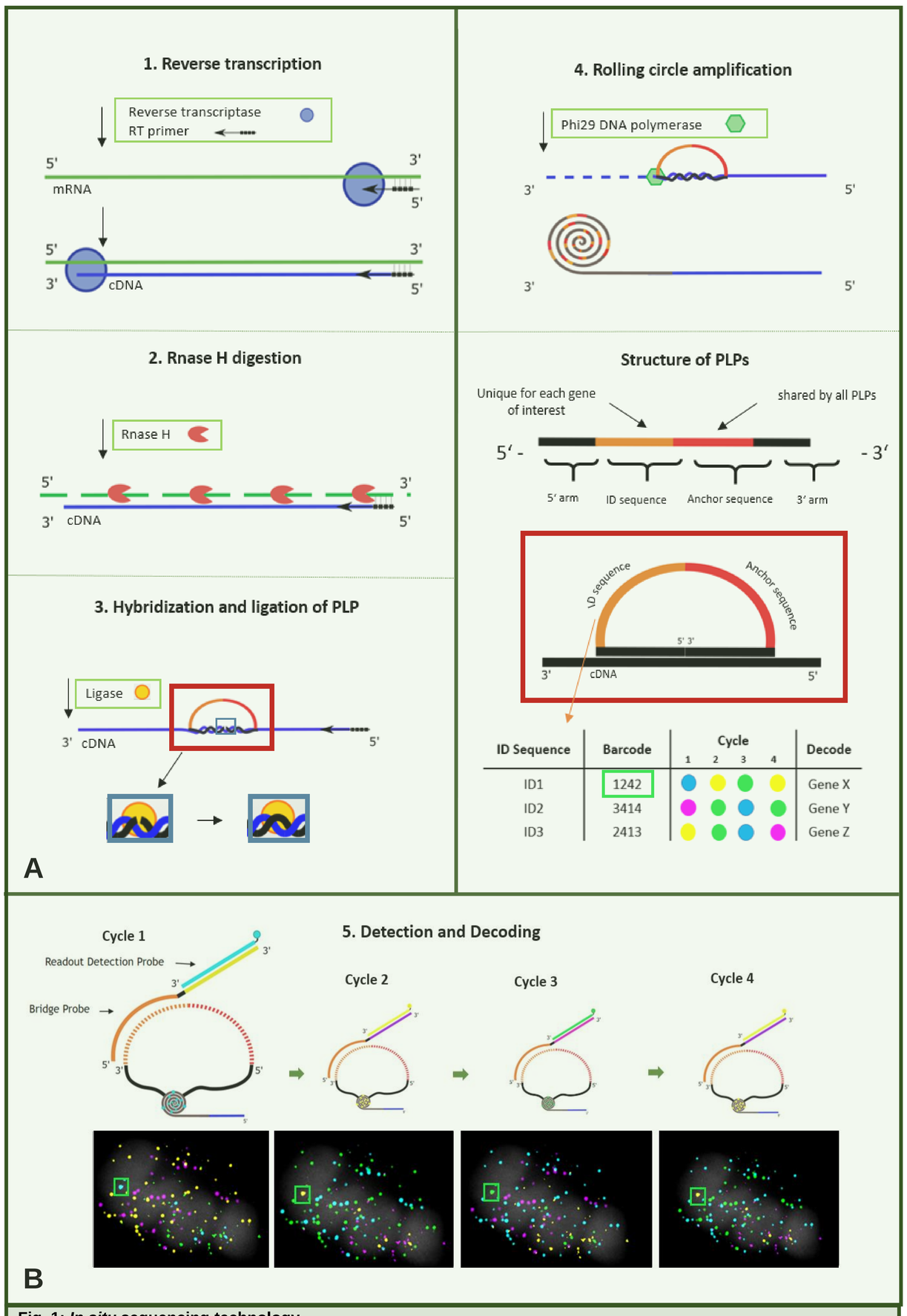

Fig. 1: *In situ* sequencing technology
(A) Overview of the *in situ* sequencing technology, the structure of the PLPs and the decoding scheme. (B) Detection and Decoding: In each cycle bridge probes are added, which bind specifically to the RCP and afterwards they are detected by adding readout detection probes that are conjugated with fluorophores.

Next steps

- Ten samples from colorectal cancer patients will be collected - **blood before surgery and tissue from the primary tumor**.
- NGS of the tumor DNA and the plasma from liquid biopsy will be performed.
- ***In situ* sequencing technology** with the designed panel of 260 gens will be performed.
- **This data will help bridging the gap between sequencing information and the histological context.**

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