ImmunoSpot Layer Imaging of Cell Excretions (iSLICE) technology for presence and strength of binding of IgM, IgG and IgA antibodies against SARS-CoV-2 during CoViD-19 infection

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ABSTRACT

During the development of ImmunoSpot Layer Imaging of Cell Excretions (iSLICE) technology, originally Surface Plasmon Resonance imaging (SPRi) technology was prepared for detecting the secretion levels of cancer cells. However, after the CoViD-19 outbreak the project partners decided to change their iSLICE focus to determine the strength of binding of IgG, IgM and IgA against SARS-CoV-2 antigens in sera of CoViD-19 patients. The high-throughput SPRi assay provides additional insights in the immune status of patients recovering from CoViD-19. Our work funded by ATTRACT led to three patent applications and prior to a high impact journal submission a preprint paper on the online Research Square platform.

Keywords: Surface plasmon resonance imaging, antibody isotype detection, coronavirus, SARS CoV-2, serum analysis, strength of binding, biosensor

1. INTRODUCTION

Our screening technology called “iSLICE” (immuno Spot Layer Imaging of Cell Excretions) applies chip array and 3D-imaging technology developed at the Medical Cell BioPhysics group (MCBP) at the University of Twente and the Nanobio group at Saxion University of Applied Sciences. Xantec Bioanalytics (Düsseldorf, Germany) as partner developed the surface chemistry for iSLICE. In iSLICE, a relevant panel of different molecules (antibodies at hybridoma cells) can be immobilized in layers and the secreted antibodies could be detected as immunoSpots. Most current serological tests measure the specific concentration of antibodies in human sera against e.g. a certain infectious disease and we have shown that the iSLICE technology was able to detect that.

After the COVID-19 outbreak it appeared that our technology for iSLICE was really suitable for detecting specific antibodies against SARS-Cov2 antigen. In contrast to the standard polymerase chain reaction (PCR) test, serologic assays demonstrate the presence of an immune reaction against the virus through detection of immunoglobulins directed against SARS-CoV-2 structural proteins [1]. The quality of the immune response is not only determined by the quantity of antibodies but also by the overall strength of binding of the pool of potential neutralizing antibodies that binds to the relevant immunogenic proteins of the Corona virus.

The iSLICE technology applies high throughput Surface Plasmon Resonance imaging (SPRi) and it can be applied for the quantitative measurement of IgG, IgM and IgA antibodies binding to the RBD spike protein [2] and their apparent polyclonal affinity in sera of CoViD-19 patients. So, InterFluidics aims to contribute via the ATTRACT-iSLICE to fighting the Corona pandemic, while keeping its goals for developing breakthrough imaging technology.

2. STATE OF THE ART

Infectious diseases and the immune status of patients can be monitored using the ELISpot principle by applying single cell secretions of Peripheral Blood Mononuclear Cells (PBMC). These PBMC samples are indicative to the response of the patient’s immune system. Initially in the iSLICE proposal, the project partners aimed to develop imaging technology to detect these secretions of PBMC’s using ELISpot and SPRi for automated real-time detection.

Then, in March 2020, the Corona outbreak was a fact. After the lock-down the team could not cultivate cells anymore and the project was urged to stop. However, in discussion with our partners we agreed a new direction of the iSLICE research.

The iSLICE approach enables to measure antibodies from single cells but also the specific antibodies in serum produced by B cells. The immune system of a CoViD-19 patient produces antibodies to SARS-CoV-2 within days to a few weeks following viral infection [1]. Serological antibody testing is essential to get an indication whether or not an individual has been infected with SARS-CoV-2. Antibody detection is typically performed using ELISA or related automated immuno-assays.

An attractive alternative for an antibody detection method was the technology we applied in iSLICE: surface plasmon resonance imaging (SPRi) technology. SPRi is a label-free sensing technique that is highly sensitive enabling the quantitative and qualitative interaction between biomolecules, such as the interaction between antibodies and their respective antigens [3]. Additionally, the strength of binding measured by the off-rate can be determined in a single assay to obtain a measure of the quality of the total polyclonal antibody response.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

By changing focus to COVID-19 immunity testing the iSLICE principle as originally proposed generated a COVID19 breakthrough. Three patent applications were filed for the iSLICE inventions and the latest was Corona related. The impact of COVID19 motivated the iSLICE team to work on characterizing immunity profiles of patients. The following three ~~initiatives resulted in~~ breakthroughs were achieved ~~initiatives~~.

1. An iSLICE device that consists of at least a reversed top capture layer clamped on a bottom layer with in between the cells. This so called DISPLACE technology (Dual immunoSpot Print Layer Array of Cell Excretions) applies two layers (regular and reversed) for detection. E.g. the bottom layer can be used to monitor cell growth with SPRimaging while the top layer can be used to measure the secreted molecules from the cells or vice versa. In another approach the bottom layer can both be used in two layers of coupled antibodies as a capture layer of cells and secretion layer for detection using SPRi.
2. Method for real time monitoring the deposition of a ligand on an evanescent field biosensor, using a micro array spotting device that consists of a support body with wells or channels for printing various proteins, antibodies, cells etc on a sensor surface for the formation of assays, biochips, biosensors and cell cultures. The spotter enables to deposit at an ultra-low volume while still no automation is required to spot ligands on top of a biosensor.
3. Method for measuring the strength of binding of isotypes of antibodies using SPR imaging and method to reduce the number of false positives characterized by:

- A print of samples whereby the print is exposed partly to an elution buffer.
- Followed by measurement of eluted and non-eluted prints that are simultaneously exposed to concatenated injections of anti- isotype antibodies.
- The difference between total and eluted part can be determined as additional parameter to the concentrations of the isotype antibodies.

These three patent applications were necessary to address break-through imaging technology for cell secretions and strength of binding detection of IgM, IgG and IgA isotypes for COVID-19 patients. Currently (Aug, 2020) the clinical value of this approach is investigated and a manuscript describing the results is being prepared. If the strength of binding parameter is a most important parameter to assess the quality of the immune status of a COVID19 patient then our technology will gain a great value. Vaccine developers will definitely have interest in our technology as our outcome of the ATTRACT project.

4. PROJECT RESULTS

For SPRi measurements the multiplex SPR imaging instrument (IBIS MX96, IBIS Technologies, Enschede, the Netherlands) and the Carterra LSA platform (Salt Lake City UT, US) were used with an installed sensor prism (HC30M) of our iSLICE project partner Xantec Bioanalytics (Düsseldorf, Germany). Similar results for both instruments were obtained with this sensor surface. After washing the sensor chip spotted with patient sera in two concatenated sessions, the sensor was first incubated with 50x diluted goat-anti-human-IgM (aIgM ~4 mg/ml, 20-S5170 GND1-D0 Fitzgerald) in running buffer (200 µl for one run) and the second a 100x diluted goat-anti-human-IgG (aIgG-Fc ~8 mg/ml, 20-S1211G001-S4 Fitzgerald) in SPRi running buffer. The third injection was with a 100x diluted goat-anti-human-IgA (aIgA ~7 mg/ml, 20-S1111G000-S4 Fitzgerald). The Rmax value was determined using a special biphasic fit algorithm (InterFluidics, Haaksbergen, The Netherlands). This software tool programmed using Microsoft 'R-Studio' allows calculating the data on both SPR imagers. If the curve did not show an exponential behaviour (e.g. negative samples) then a linear fit was applied and the average value of the linear fit was determined.

In total 48 selected serum samples were spotted in duplicate in a single run on the by Xantec coupled HC30M RBD variant 3 sensor prism surface in 4 dilutions (1:50, 1:100, 1:200 and 1:400) to generate a 384-array. During the spotting process, the binding signals are followed for 15 minutes and each serum sample was measured 8 times at these 4 dilutions.

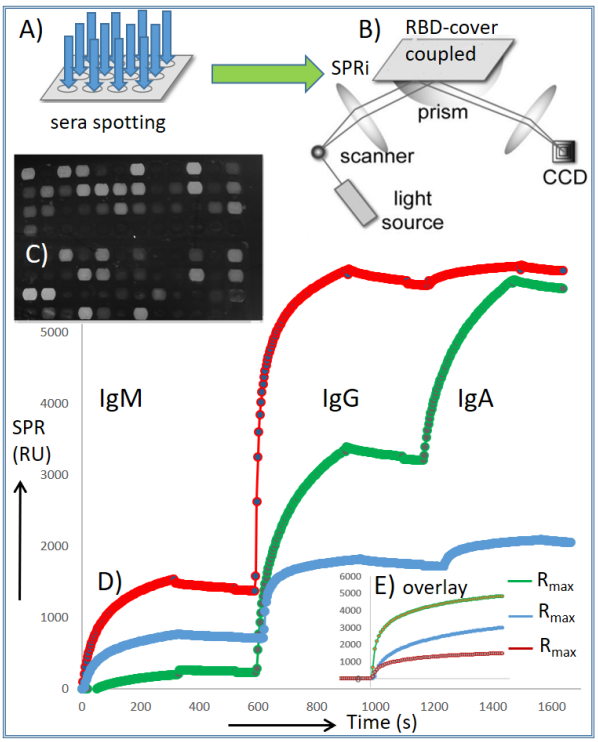

Fig. 1. Principle of the anti-SARS-CoV-2 immune globulins SPRi assay. The process of spotting the sera (A) to an RBD coupled surface in the MX96 SPRi instrument (B) resulted in a SPRi reflectivity image (C). In (D) the sensorgram is shown of injections of three positive antisera to determine the response of IgM, IgG and IgA antibodies. In panel (E) an overlay of the injections of the anti-IgM, IgA and IgG antibodies of a single spotted serum is presented for calculating the Rmax values of the IgM, IgG and IgA binding, after zeroing, aligning the sensorgram.

Following the spotting process, a 5 min injection of the free Receptor Binding Protein (RBD) (15 µg/ml) in dilution buffer resulted in sufficient dissociation of the anti-RBD antibodies. For all 384 spots, the global dissociation- or global off-rate constant can be calculated. The final step consists of sequential injections of solutions of anti-IgM, anti-IgG and anti-IgA antibodies. The ratio of bound immunoglobulins can be calculated by determining the R_{max} values from the anti-isotype antibodies binding signals. The R_{max} value has a direct relation with the concentration of anti-RBD antibodies in serum. Our iSLICE project partner Xantec Bioanalytics decided to fabricate the Corona sensor prism under validated GMP production circumstances. Recently (July 2020) **Xantec launched this sensor** in the market for facilitating COVID19 research using SPR technology.

Strength of binding measurement of anti-SARS-CoV-2 Spike RBD IgG, IgA and IgM

To reduce the rebinding effect of dissociating molecules, we added free RBD in a concentration of 15 µg /ml to the running buffer. In 5 minutes, we observed a mixed degree

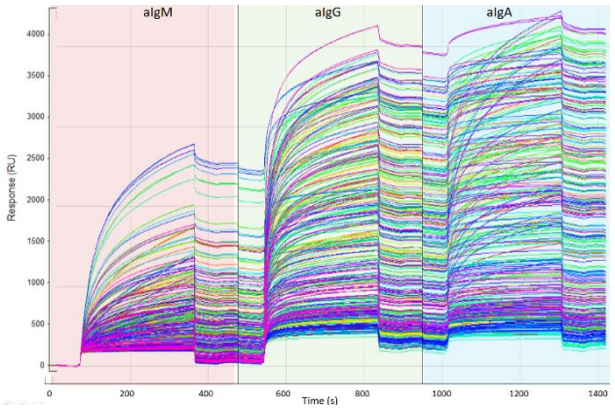

Fig. 2. Raw non-referenced, non-zeroed sensorgrams of 384 spots (96 patients) after concatenated injections of anti-IgM, anti-IgG and anti-IgA antibodies using the LSA SPR imager. Huge signals are obtained from positive tested patients. The immunity condition of COVID-19 patients can be monitored in a measurement of 1400 seconds. The total test can be carried out in less than an hour.

of dissociation of the various single and longitudinal samples and the dissociation or off-rate constant can be calculated and plotted as a function of the days of symptoms onset. (Figure 3).

During the recovery process of the disease, we observed a lower off-rate indicating that the avidity or quality of the antibodies improves. Recently antiNucleocapsid antibodies show a reverse correlation with convalescence versus deceased patients [4]. So, the patients are producing a better-quality repertoire of polyclonal anti-RBD antibodies over time. For all our longitudinal samples this trend in off-rate is observed (strength of binding becomes better). We developed a new method to discriminate between weak and strong binding antibodies and this was realized and described in a patent application filed on Aug 19th, 2020. Our method for profiling the immunity in terms of isotype concentration and strength of binding enables to reveal the quality of the immune response of COVID-19 patients.

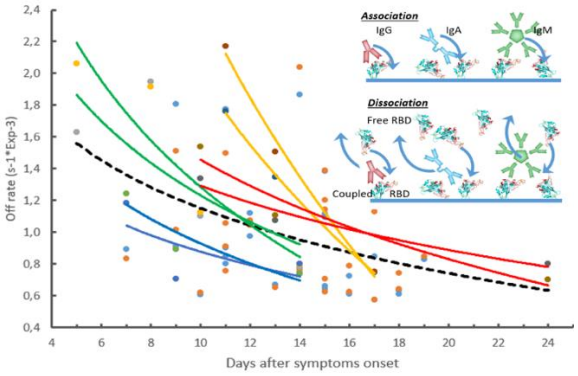

Fig. 3. The dissociation constant of the anti-RBD antibodies as function of the days after symptoms onset. The black dashed line is the overall trend line of all measured samples. The colored lines connect four longitudinal samples in duplicate.

Kinetically measuring the affinity using the ligand in the buffer is a classical and well-known way for better measuring the dissociation rate of the bound molecules to the same ligand. However, we found a better approach for assessing the immunity of patients and claimed this in a patent application.

A huge difference was seen between patients with weakly bound antibodies and strongly bound antibodies. The method discriminates also between the quality of binding of the individual isotypes IgM, IgG and IgA. The table shows the huge data generation on the immunity of patients. The data can be generated in less than an hour.

Table. *Results obtained with the new method. The eluted antibody with respect to the total is shown in the third column section. An affinity maturation effect is measured on the colored longitudinal samples. A dramatic improvement of measuring false-positives has been realized. (noted as neg).*

Full MGA respons					Eluted MGA response					Polyclonal avidity % after elution					
Naam	IgM	IgG	IgA	Totaal	Naam	IgM	IgG	IgA	Totaal	Ratio %	IgM	IgG	IgA	Totaal	
[001] 1	288	2613	1704	4605	[049] 1	162	1559	1493	3214	[001] 1	56	60	88	70	
[002] 2	25	97	151	273	[050] 2	10	11	6	27 neg	[002] 2	40	11	4	10	
[003] 3	780	1373	2887	5040	[051] 3	337	430	982	1749	[003] 3	43	31	34	35	
[004] 4	2914	2622	291	5827	[052] 4	328	1190	15	1533	[004] 4	11	45	5	26	
[005] 5	134	44	13	191	[053] 5	34	10	5	49 neg	[005] 5	25	23	38	26	
[006] 6	41	214	31	286	[054] 6	4	6	5	15 neg	[006] 6	10	3	16	5	
[007] 7	5191	2479	260	7930	[055] 7	888	1410	53	2351	[007] 7	17	57	20	30	
[008] 53	1118	3225	135	4478	[056] 53	471	1547	68	2086	[008] 53	42	48	50	47	
[009] 60	2851	2778	188	5817	[057] 60	987	1189	169	2345	[009] 60	35	43	90	40	
[010] 10	738	3301	1159	5198	[058] 10	248	1651	871	2770	[010] 10	34	50	75	53	
[011] 11	200	247	242	689	[059] 11	74	42	61	177	[011] 11	37	17	25	26	11
[012] 12	841	770	484	2095	[060] 12	356	271	182	809	[012] 12	42	35	38	39	
[013] 13	830	1798	331	2959	[061] 13	452	806	181	1439	[013] 13	54	45	55	49	
[014] 14	598	2705	150	3453	[062] 14	226	1486	55	1767	[014] 14	38	55	37	51	
[015] 15	381	907	3046	4334	[063] 15	140	232	2083	2455	[015] 15	37	26	68	57	15
[016] 16	982	2246	2056	5284	[064] 16	246	898	1834	2978	[016] 16	25	40	89	56	
[017] 17	832	2625	1577	5034	[065] 17	198	1334	1601	3133	[017] 17	24	51	102	62	
[018] 18	1970	1993	539	4502	[066] 18	874	985	376	2235	[018] 18	44	49	70	50	18
[019] 19	1916	2065	536	4517	[067] 19	628	848	299	1775	[019] 19	33	41	56	39	
[020] 20	119	509	327	955	[068] 20	63	99	73	235	[020] 20	53	19	22	25	20
[021] 21	467	1687	413	2567	[069] 21	165	450	200	815	[021] 21	35	27	48	32	
[022] 22	653	3081	1161	4895	[070] 22	295	1427	961	2683	[022] 22	45	46	83	55	
[023] 23	2230	264	532	3026	[071] 23	1173	116	274	1563	[023] 23	53	44	52	52	23
[024] 24	3979	1150	519	5648	[072] 24	2148	514	381	3043	[024] 24	54	45	73	54	
[025] 25	1613	1098	1248	3959	[073] 25	905	497	794	2196	[025] 25	56	45	64	55	
[026] 26	14	8	8	30	[074] 26	4	2	2	8 neg	[026] 26	29	25	25	27	26
[027] 27	340	332	142	814	[075] 27	134	75	3	212	[027] 27	39	23	2	26	
[028] 28	488	1267	303	2058	[076] 28	223	388	71	682	[028] 28	46	31	23	33	
[029] 29	642	1944	298	2884	[077] 29	247	922	197	1366	[029] 29	38	47	66	47	
[030] 30	512	2378	350	3240	[078] 30	266	1320	242	1828	[030] 30	52	56	69	56	
[031] 31	532	2039	161	2732	[079] 31	324	676	56	1056	[031] 31	61	33	35	39	
[032] 32	2108	144	269	2521	[080] 32	1489	56	122	1667	[032] 32	71	39	45	66	
[033] 33	851	510	1973	3334	[081] 33	216	78	646	940	[033] 33	25	15	33	28	
[034] 34	7	89	189	285	[082] 34	4	10	46	60 neg	[034] 34	57	11	24	21	
[035] 35	1974	2605	290	4869	[083] 35	395	990	129	1514	[035] 35	20	38	44	31	
[036] 36	4688	1196	425	6309	[084] 36	4031	596	400	5027	[036] 36	86	50	94	80	
[037] 92	768	2105	312	3185	[085] 92	760	1574	176	2510	[037] 92	99	75	56	79	
[038] 103	76	1493	2878	4447	[086] 103	75	918	1882	2875	[038] 103	99	61	65	65	
[039] 63	1122	1086	412	2620	[087] 63	252	331	82	665	[039] 63	22	30	20	25	
[040] 64	188	1143	3176	4507	[088] 64	117	520	1131	1768	[040] 64	62	45	36	39	
[041] 65	2734	1491	850	5075	[089] 65	1067	839	528	2434	[041] 65	39	56	62	48	
[042] 66	46	69	77	192	[090] 66	8	4	4	16 neg	[042] 66	17	6	5	8	
[043] 67	32	108	96	236	[091] 67	11	3	4	18 neg	[043] 67	34	3	4	8	
[044] 107	117	261	3116	3494	[092] 107	3	5	7	15 neg	[044] 107	3	2	0	0	
[045] 110	669	2150	2261	5080	[093] 110	261	328	2260	2849	[045] 110	39	15	100	56	
[046] 70	296	3577	106	3979	[094] 70	206	1783	31	2020	[046] 70	70	50	29	51	
[047] 71	897	945	120	1962	[095] 71	360	316	35	711	[047] 71	40	33	29	36	
[048] 72	1357	3332	493	5182	[096] 72	513	2488	490	3491	[048] 72	38	75	99	67	

FUTURE PROJECT VISION

The iSLICE project was the basis for start-up company InterFluidics and the goal changed to COVID19 testing. The new parameter 'strength of binding' is an additional parameter to the measured concentration of IgM, IgG and IgA antibodies against Corona virus antigens. [5]. InterFluidics wants to continue this route for creating impact by building a dedicated clinical SPR-imager, when the clinical correlation of convalescent and deceased (dying) patients can uniquely be measured with SPRimaging technology. Hopefully, ATTRACT Phase2 enables to collect partners to contribute to solutions for the COVID19 pandemic using this technology.

4.1. Technology Scaling

Two commercial SPR-imaging instruments were used for the "strength of antibody binding" tests. However, these instruments of 250 k€ (IBIS) and 500 k€ (Carterra) are both not applicable to distribute the test in the clinical market. A fully dedicated lower priced (~50k€) SPR-imaging instrument should be developed to test the concentration and quality of antibodies from COVID-19 patients. InterFluidics is able to show the roadmap for this dedicated clinical instrument. First, the clinical outcome of the parameter "strength of antibody binding" should be determined and approved to be of great clinical relevance. TRL8 for the current **commercial SPR-imagers is** possible during ATTRACT phase 2. Clinical immunologists, epidemiologists and virologists should assess further the relevance of the parameter that is measured and developed with the iSLICE/ATTRACT technology. The quality of COVID-19 antibodies should be of importance to assess immune responses to Corona vaccines, which will enter the market, as expected, in the beginning of 2021. Then the three patent applications of InterFluidcs will gain great value. InterFluidics will then license its technology to other parties enabling very fast market introduction **of the dedicated SPR imaging technology for COVID19**.

4.2. Project Synergies and Outreach

InterFluidics follows two routes, bottom up and top-down for implementing the tests in the market. It applies now the top-down approach, the commercial available research instruments for creating awareness and importance of performing these tests for Covid-19 patients. These very expensive research instruments enable to perform many more applications, which are not necessary for Covid19 testing. InterFluidics' partner Xantec is selling the specific Corona sensor surfaces on these imagers already since July 2020. All conditions including patents are met to build a dedicated lower-cost clinical diagnostic instrument for measuring the immune status of Corona patients and for cell secretion detection. In this bottom-up way, InterFluidics is able to run along the TRL levels of development very fast and starts at about TRL level 6. Attract Phase 2 is of great importance to follow this route and attract other partners for the Clinical SPRimager. InterFluidics is involved in an ECsens initiative for Phase2, but this has a different goal, but applies the new proposed SPRimager similarly.

4.3. Technology application and demonstration cases

The iSLICE ATTRACT project is for InterFluidics of great value when the condition of clinical relevance is proven. Now in an extensive test with various hospitals that deliver longitudinal samples (samples from a single person taken at different times), we are investigating the parameter "strength of binding" for correlation with other patient data. After validation of its relevance, InterFluidics is looking for partners for implementing the technology in this specific health, demographic change and well-being market. Contributing to the COVID-19 pandemic is a clear and relevant goal.

4.4. Technology commercialization

When the clinical relevance of the antibody quality parameter of COVID-19 patients has been established then investors are attracted to financially contribute to the clinical COVID-19 SPR imager production. The company Vysens B.V. showed already interest in developing the SPRi instrument. Since July 2020, we discuss the commercialization trajectory twice a week. InterFluidics is now preparing a business plan.

4.5. Envisioned risks

If the parameter "strength of binding" of IgG, IgM and IgA is of less importance, then the provoked effort is less attractive. The coming period is crucial to accelerate or not. The iSLICE inventions possessed by InterFluidics are crucial to develop the instrument, but other SPR companies can change their focus too. The risk is further that the development takes longer than expected and therefore more costly than budgeted. Then we forecast an additional investment and effort to realize this.

4.6. Liaison with Student Teams and Socio-Economic Study

InterFluidics has partly its base at the labs of the Medical BioPhysics Group based at the University of Twente and the NanoBio group of Saxion Applied University and students from both Twente and Saxion will be involved for developing and improving the clinical prototype. In addition, software tools and dedicated algorithms will be developed for clearly presenting the results to physicians and clinicians.

This study was performed in accordance with the guidelines for sharing of patient data of observational scientific research in emergency situations as issued by the Commission on Codes of Conduct of the Foundation Federation of Dutch Medical Scientific Societies (https://www.federa.org/federa-english).

5. ACKNOWLEDGEMENT

The Dutch Research Council (NWO) is acknowledged for support of sensors and reagents. Thanks to Leontine Mulder of the Department of Clinical Chemistry, Medisch Spectrum Twente, Enschede who allowed us to apply Covid19 patient samples. This project has received funding from the ATTRACT project funded by the EC under Grant Agreement 777222.

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