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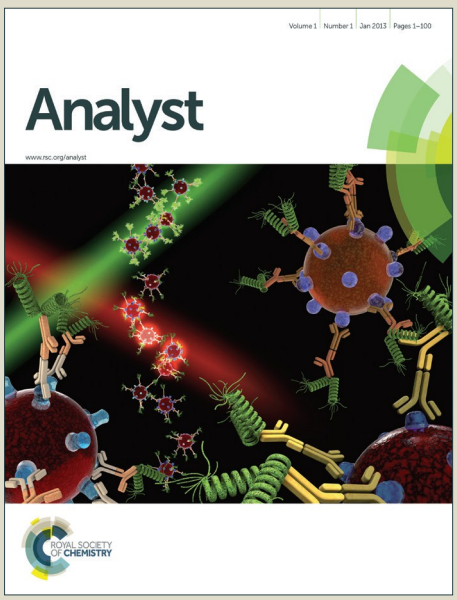

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A Universal Tumor Cell Isolation Method Enabled by Fibrin-coated Microchannels

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We report a simple but effective strategy to capture tumor cells using fibrin-immobilized microchannels. It is a universal method since it shows an ability of capturing both epithelial and mesenchymal tumor cells. The cell capture efficiency is up to 90%.

Analysis of circulating tumor cells (CTCs) has important clinical significance in cancer diagnosis, treatment monitoring, and prediction of overall survival rate of patients.[1-3] Researchers have demonstrated that the CTC levels are related to prognosis in patients with cancer of breast, prostate, lung, and colon.[2, 4-6] Microfluidic devices have been applied for capturing CTCs mainly by using EpCAM antibody that interacts with tumor cells in microchannels.[7-11] However, it has been reported that the level of EpCAM expression varied among CTCs, including patients with hepatocellular carcinoma,[12, 13] metastatic breast cancer disease,[14] and melanoma[15]. In addition, the surface expression of biomarkers on CTCs became heterogeneous due to the epithelial-mesenchymal transition (in general, epithelial cells possess EpCAM whereas mesenchymal cells have no or little EpCAM).[3] Thus, capture of CTCs based on their EpCAM expression can result in a substantial loss of informative CTCs.

Clinical applications require a method that can effectively capture CTCs from all types of cancer to build universal standard for early diagnostics and monitoring progress of treatment. Technologies using tumor cells' own properties (for example, invasive property,[16, 17] specific antigen-secreting[18-20]) for cell capture could be advantageous.

Fibrin is a fibrous protein, generated by the polymerization of fibrinogen with the protease thrombin. The fibrin polymer interacts with platelets to form a hemostatic clot on a wound site.[21] In cancer metastasis study, it has been proved that fibrin polymers can bind to the receptors on tumor cell surface.[22 23] As a result, we explored this property for tumor cell isolation. Note that fibrin-coated microchannels have been used to evaluate thrombosis and three-dimensional network structures.[24-27]

We immobilized fibrin polymers on the surface of the microchannels in a microfluidic device for capturing tumor cells as shown in Figure 1. Our results indicate that capture efficiency of up to 90% can be obtained.

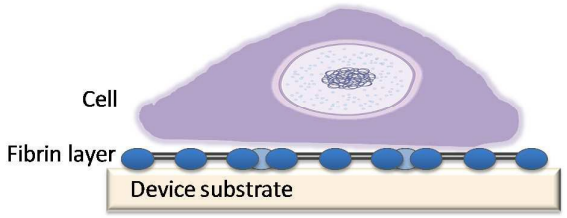

Figure 1. Schematic showing the fibrin-based tumor cell capture. The drawing is not to scale.

We hypothesized that microfluidic devices immobilized with fibrin can capture tumor cells from a sample. To verify the hypothesis, we performed cell capture experiments targeting acute lymphoblastic leukemia cells (CCRF-CEM cells) and human pancreatic cells (PAN-1 and MIAPaCa-2). For the proof-of-concept study, we fabricated a microfluidic device consisting of one inlet, connecting to eight parallel channels via consecutive bifurcation, and one outlet, as we reported previously.[28] Before use, the channel surface of the device was modified through thrombin-fibrinogen reaction. To generate a uniform layer of fibrin on the glass substrate, the reaction was optimized to form a thin layer of fibrin to achieve strong capture of tumor cell (Supplementary information). In brief, the device was first washed by ethanol and 0.1 M citric acid-sodium citrate buffer (CA buffer, pH 6.6). It was then incubated with thrombin for 30 min, which was adsorbed to

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glass substrate. After washed by the CA buffer, a fibrinogen solution (pH 6.6, containing 25 mM $CaCl_2$) was introduced and incubated for 45 min. Finally, PBS buffer was introduced into the device to stop the reaction. Since the reaction is highly dependent on the pH condition and mediated by Ca^{2+}, it would stop when the PBS buffer was introduced.[29-31] The generation of fibrin polymer inside the microchannels was confirmed by Coomassie Blue staining (Supplementary information). The thickness of the fibrin gel is from 10 nm to 1000 nm according to literature.[32]

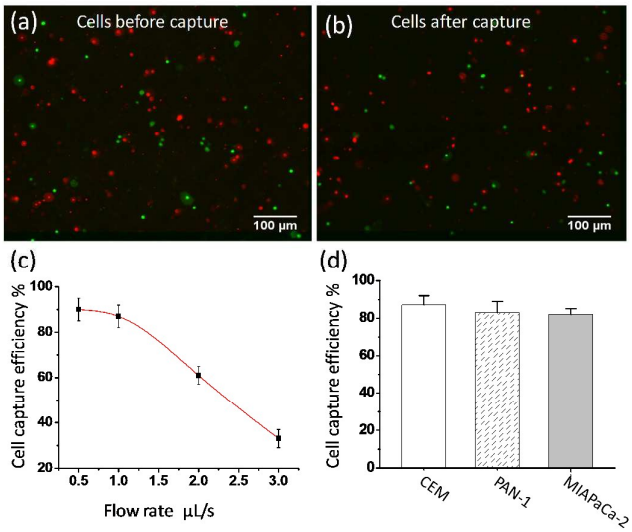

Figure 2. (a, b) Representative image of the target CCRF-CEM (green) and MIAPaCa-2 (red) before capture (a), and after capture (b). (c) The effects of the flow rate on the cell capture efficiency. (d) Performance of different cells with different EpCAM expressions: CCRF-CEM cells (EpCAM negative); PAN-1 (EpCAM positive); MIAPaCa-2 (EpCAM negative).

After surface modification, the device was evaluated for cell capture. Figure 2a shows the composition of a cell mixture consisting of two EpCAM negative cells, CCRF-CEM cells and MIAPaCa-2 cells. CCRF-CEM cells and MIAPaCa-2 cells were pre-stained respectively with DiD (green) and Vybrant DiI (red) before introducing into the device. After going through the device, a similar composition of two types of cells was observed as shown in Figure 2b, indicating that both types of cells were captured by fibrin on channel surfaces.

To achieve high throughput of the platform with minimum time required for cell capture, we performed the effects of flow conditions on cell capture in the device. We examined the cell capture efficiency for 10^5/mL target CCRF-CEM cells in PBS buffer at a flow rate ranging from 0.5 μL/s to 3.0 μL/s. The cell capture efficiency was calculated by dividing the number of target cells captured by the number of target cells introduced into the device. As shown in Figure 2c, the capture efficiency of CCRF-CEM cells decreased as the flow rate increased. Flow rates less than 1.0 μL/s enabled high capture efficiency, which significantly decreased when flow went was higher than 1.0 μL/s. It may due to destruction of the fibrin gel by shear stress or the shear force at a high flow rate, which is too strong for cells to remain captured. As a result, we used 1.0 μL/s as the flow rate for the remaining cell capture experiments.

We then compared cell capture efficiency among cells with different EpCAM expression levels. CCRF-CEM cells are EpCAM negative; human pancreatic cell lines, PAN-1, are EpCAM positive; MIAPaCa-2 cells are EpCAM negative.[28, 33, 34] At a flow rate of 1.0 μL/s, the fibrin gel immobilized in a microfluidic device captured both EpCAM positive and EpCAM negative cells with capture efficiency around 90% as shown in Figure 2d.

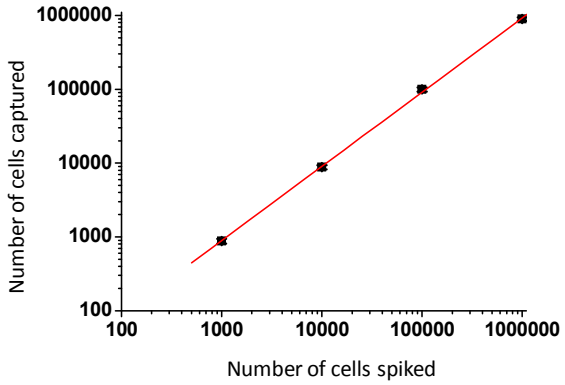

Figure 3. Calibration plot of cancer cell (CCRF-CEM cells) capture from PBS buffer solution with different cell concentrations at a flow rate of 1.0 μL/s; solid lines represent linear fitting. RSDs obtained from each the concentration are all below 10%.

To study the performance of fibrin for capturing different concentrations of targeting cells, we prepared a cell suspension ranging from 10^3 to 10^6 cells/mL of CCRF-CEM cells in PBS buffer. As shown in Figure 3, the fibrin-immobilized microfluidic device had capture efficiency of about 90% for all cases at the flow rate of 1.0 μL/s, indicating the stability and reliability of the platform.

To evaluate the specificity of fibrin for capturing tumor cells, we studied the cell capture of human leukemia cells in blood samples. Researches have showed that different adhesive proteins and platelet membrane proteins are involved in aggregation, which depends on the shear stress condition and the concentration of divalent cations.[35] Platelets from blood containing [Ca^{2+}] within normal plasma levels do not undergo low shear-induced aggregation (shear force less than 12 dyn/cm^2), whereas fibrinogen is not involved in high shear-induced aggregation (shear force above 80 dyn/cm^2).[35] With blood sample processed at a flow rate of 1 μl/s (corresponds to a wall shear force of 0.38 dyn/cm^2), we did not observe platelet aggregation. Some of red blood cells (RBC) were attached to the microchannel walls, but they were eliminated in enumeration by subsequent nucleus DNA staining using DAPI (4',6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA). We prepared a cell suspension ranging from 10^3

to 10^6 cells/mL of CCRF-CEM cells spiked in human whole blood. We found that the CCRF-CEM cells were isolated with capture efficiency at 86±4% (Supplementary information for detailed experiments). For the cell concentration of 5×10^3 cells/mL, the cell capture purity was 42±7%. The cell purity was calculated by dividing the number of target cells captured on the device by the number of total captured cells, which included both target cells and the unspecific bound cells. At a flow rate of 1.0 μL/s, the capture efficiency is compatible with either antibody or aptamer-based cell capture methods.[28, 36] For concentration of 10^3/mL CCRF-CEM cells, the fibrin-immobilized device showed sufficient selectivity of target cells over millions of white blood cells in the blood sample. For 1 mL of blood processed, ~6000 leukocytes (~0.09% of total leukocytes) were captured for spiking experiments using healthy samples.

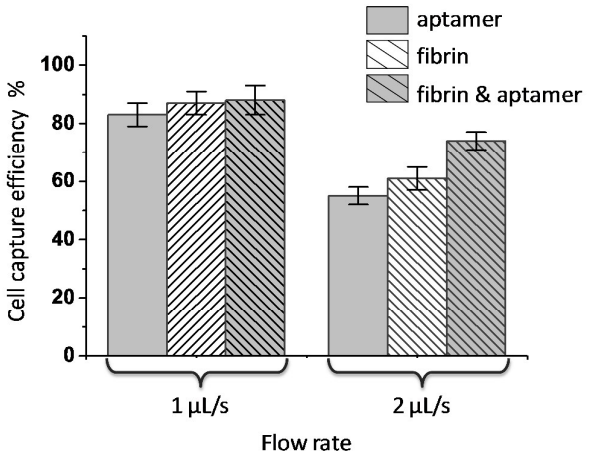

Figure 4. Cell capture comparison of aptamer alone, fibrin alone, and aptamer-fibrin combination. The capture efficiency was of 10^3/mL CCRF-CEM cells in PBS buffer. Error bars represent one standard deviation (n = 3).

Besides of utilizing the fibrin polymer gel alone for cell capture, the fibrin can be combined with antibodies or aptamers for enhanced capture efficiency of tumor cells. We added avidin in fibrinogen solution before being introduced into the device. After the thrombin-fibrinogen reaction, the surface was coated with fibrin gel as before while avidin in the gel and on the surface allow for further chemical interactions. A biotinylated aptamer, sgc8, which specifically binds with CCRF-CEM cells,[36] was then introduced into the device, followed by incubation. The aptamer was attached to the surface through avidin-biotin chemistry, forming an aptamer-fibrin gel. We compared aptamer-fibrin with aptamer alone or fibrin alone for capture cells using identical devices and conditions (Supplementary information). As shown in Figure 4, at a flow rate of 1.0 μL/s, the capture efficiency is similar for fibrin alone, aptamer alone, and the aptamer-fibrin combination gel. However, at a higher flow rate of 2.0 μL/s, the capture efficiency of aptamer-fibrin combination increased 34% and 21% over aptamer alone and fibrin alone, respectively. The decrease in the overall capture efficiency from a flow rate of 1.0 μL/s to 2.0 μL/s is in agreement with the literature because of the reduced interaction time between cells and capture agents on the surface at a higher flow rate as well as larger shear forces. The result indicates an enhanced interaction between tumor cells and the aptamer-fibrin surface, which sustains at a high flow rate. We can infer that fibrin gel can be used to immobilize other molecules such as antibodies in microfluidic devices for tumor cell capture and other applications.

Conclusions

We developed a fibrin-based microfluidic platform for tumor cell capture with high efficiency and high throughput. The thrombin-fibrinogen reaction was optimized in microchannels for effectively capture of tumor cells. It is a universal platform since it can capture both EpCAM positive and EpCAM negative tumor cells. Besides, the fibrin gel can be used for immobilization of other capturing ligands such as aptamers and antibodies.

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Notes and References

1. M. Cristofanilli, G. T. Budd, M. J. Ellis, A. Stopeck, J. Matera, M. C. Miller, J. M. Reuben, G. V. Doyle, W. J. Allard, L. W. Terstappen and D. F. Hayes, *N Engl J Med*, 2004, **351**, 781-791.
2. K. Pantel, R. H. Brakenhoff and B. Brandt, *Nat Rev Cancer*, 2008, **8**, 329-340.
3. C. L. Chaffer and R. A. Weinberg, *Science*, 2011, **331**, 1559-1564.
4. J. S. de Bono, H. I. Scher, R. B. Montgomery, C. Parker, M. C. Miller, H. Tissing, G. V. Doyle, L. W. W. M. Terstappen, K. J. Pienta and D. Raghavan, *Clin Cancer Res*, 2008, **14**, 6302-6309.
5. H. I. Scher, X. Y. Jia, J. S. de Bono, M. Fleisher, K. J. Pienta, D. Raghavan and G. Heller, *Lancet Oncology*, 2009, **10**, 233-239.
6. S. Halabi, E. J. Small, P. W. Kantoff, M. W. Kattan, E. B. Kaplan, N. A. Dawson, E. G. Levine, B. A. Blumenstein and N. J. Vogelzang, *J Clin Oncol*, 2003, **21**, 1232-1237.
7. S. Nagrath, L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G.

Tompkins, D. A. Haber and M. Toner, *Nature*, 2007, **450**, 1235-1239.
8. S. L. Stott, C. H. Hsu, D. I. Tsukrov, M. Yu, D. T. Miyamoto, B. A. Waltman, S. M. Rothenberg, A. M. Shah, M. E. Smas, G. K. Korir, F. P. Floyd, A. J. Gilman, J. B. Lord, D. Winokur, S. Springer, D. Irimia, S. Nagrath, L. V. Sequist, R. J. Lee, K. J. Isselbacher, S. Maheswaran, D. A. Haber and M. Toner, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, **107**, 18392-18397.
9. A. A. Adams, P. I. Okagbare, J. Feng, M. L. Hupert, D. Patterson, J. Gottert, R. L. McCarley, D. Nikitopoulos, M. C. Murphy and S. A. Soper, *J Am Chem Soc*, 2008, **130**, 8633-8641.
10. S. Wang, H. Wang, J. Jiao, K. J. Chen, G. E. Owens, K. Kamei, J. Sun, D. J. Sherman, C. P. Behrenbruch, H. Wu and H. R. Tseng, *Angew Chem Int Ed Engl*, 2009, **48**, 8970-8973.
11. X. Zheng, L. S. Cheung, J. A. Schroeder, L. Jiang and Y. Zohar, *Lab Chip*, 2011, **11**, 3269-3276.
12. T. Yamashita, M. Forgues, W. Wang, J. W. Kim, Q. Ye, H. Jia, A. Budhu, K. A. Zanetti, Y. Chen, L. X. Qin, Z. Y. Tang and X. W. Wang, *Cancer Res*, 2008, **68**, 1451-1461.
13. C. J. de Boer, J. H. van Krieken, C. M. Janssen-van Rhijn and S. V. Litvinov, *The Journal of pathology*, 1999, **188**, 201-206.
14. T. Fehm, O. Hoffmann, B. Aktas, S. Becker, E. F. Solomayer, D. Wallwiener, R. Kimmig and S. Kasimir-Bauer, *Breast Cancer Res*, 2009, **11**, R59.
15. J. P. Thiery, *Nat Rev Cancer*, 2002, **2**, 442-454.
16. H. Wang, Y. Hara, X. Liu, J. M. Reuben, Y. Xie, H. Xu, G. Bu, Y. Pei, V. Gupta and X. Wu, *Oncotarget*, 2015.
17. T. Fan, Q. Zhao, J. J. Chen, W. T. Chen and M. L. Pearl, *Gynecol Oncol*, 2009, **112**, 185-191.
18. A. Jin, T. Ozawa, K. Tajiri, T. Obata, S. Kondo, K. Kinoshita, S. Kadowaki, K. Takahashi, T. Sugiyama, H. Kishi and A. Muraguchi, *Nature Medicine*, 2009, **15**, 1088-U1146.
19. C. Alix-Panabieres, X. Rebillard, J. P. Brouillet, E. Barbotte, F. Iborra, B. Segui, T. Maudelonde, C. Jolivet-Reynaud and J. P. Vendrell, *Clinical chemistry*, 2005, **51**, 1538-1541.
20. C. Alix-Panabieres, J. P. Vendrell, O. Pelle, X. Rebillard, S. Riethdorf, V. Muller, M. Fabbro and K. Pantel, *Clinical chemistry*, 2007, **53**, 537-539.
21. B. E. Kehrel, *Hamostaseologie*, 2003, **23**, 149-158.
22. J. S. Palumbo, K. E. Talmage, J. V. Massari, C. M. La Jeunesse, M. J. Flick, K. W. Kombrinck, M. Jirouskova and J. L. Degen, *Blood*, 2005, **105**, 178-185.
23. F. W. Schardt, B. Schmausser and E. Bachmann, *Histol Histopathol*, 2013, **28**, 993-998.
24. T. V. Colace, G. W. Tormoen, O. J. T. McCarty and S. L. Diamond, *Annu Rev Biomed Eng*, 2013, **15**, 283-303.
25. J. H. Yeon, H. R. Ryu, M. Chung, Q. P. Hu and N. L. Jeon, *Lab Chip*, 2012, **12**, 2815-2822.
26. M. M. Dudek, N. J. Kent, P. Gu, Z. H. Fan and A. J. Killard, *Analyst*, 2011, **136**, 1816-1825.
27. C. J. N. Brian R. Branchford, Keith B. Neeves, Jorge Di Paola, *Thrombosis Research*, 2015, **136**, 13-19.
28. W. A. Sheng, O. O. Ogunwobi, T. Chen, J. L. Zhang, T. J. George, C. Liu and Z. H. Fan, *Lab Chip*, 2014, **14**, 89-98.
29. A. Mathur, W. A. Schlapkohl and E. Di Cera, *Biochemistry*, 1993, **32**, 7568-7573.
30. H. Zhao, L. Ma, J. Zhou, Z. Mao, C. Gao and J. Shen, *Biomed Mater*, 2008, **3**, 015001.
31. S. W. Kang, J. S. Kim, K. S. Park, B. H. Cha, J. H. Shim, J. Y. Kim, D. W. Cho, J. W. Rhie and S. H. Lee, *Bone*, 2011, **48**, 298-306.
32. T. Riedel, E. Brynda, J. E. Dyr and M. Houska, *J Biomed Mater Res A*, 2009, **88**, 437-447.
33. L. Ren-Heidenreich, P. A. Davol, N. M. Kouttab, G. J. Elfenbein and L. G. Lum, *Cancer*, 2004, **100**, 1095-1103.
34. C. Huang, J. P. Smith, T. N. Saha, A. D. Rhim and B. J. Kirby, *Biomicrofluidics*, 2014, **8**, 044107.
35. Y. Ikeda, M. Handa, K. Kawano, T. Kamata, M. Murata, Y. Araki, H. Anbo, Y. Kawai, K. Watanabe, I. Itagaki and et al., *J Clin Invest*, 1991, **87**, 1234-1240.
36. W. A. Sheng, T. Chen, R. Katnath, X. L. Xiong, W. H. Tan and Z. H. Fan, *Analytical Chemistry*, 2012, **84**, 4199-4206.