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Abstract. Mesenchymal stem cell (MSC) homing and integration into tumors are under evaluation for clinical application. This approach requires the identification of conditions for optimal tumor invasion. We describe a tool for the *in vitro* comparison of parameters influencing invasion. Human MSC added to experimental tumor spheroids variably migrates toward the center of the structure. To determine MSC distribution inside the three-dimensional specimen, spatial analysis was performed using selective plane illumination microscopy. A standardized method to quantify and compare the invasion potential of variably treated MSC into experimental tumor environments allows efficient screening for optimizing conditions. © The Authors.

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Mesenchymal stem cells (MSCs) are actively recruited to tumor microenvironments where they can promote tumor growth. Because tumors are seen by the body as chronic wounds, they attract MSC in an attempt to effect repair.^{1,2} This property has been exploited to generate engineered MSC (eMSC) that acts as cellular vehicles carrying therapy genes deep into tumor environments. An early version of the eMSC is being evaluated in clinical trials for the treatment of gastroenterological malignancies.^{3,4} Studies have shown that different preparations of eMSC

can show variability in their ability to invade tumors. This has driven the search for methods that would allow for a systematic evaluation of the effects of different variables on the efficiency of eMSC to invade tumors.

In vitro derived tumor spheroids have been shown to recapitulate features of their solid tumor counterparts, specifically, aspects of intervascular tumor microregions.^{5,6} They provide insight into tumor composition, growth, and physiology offering an easily manageable tool for cancer studies, including a platform for evaluating the ability of eMSC to invade tumor environments.

We developed an *in vitro* tool for quantification of MSC invasion into tumor spheroids. It uses selective plane illumination microscopy (SPIM) which allows three-dimensional (3-D) image acquisition and rapid-robust screening of parameters impacting MSC infiltration into tumor environments.

Human hepatocellular carcinoma-cell (HUH7) spheroids were used as tumor models. Primary human MSCs extracted from bone marrow (provided by apceth GmbH, Munich, Germany) were stained with 1 μ M CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes, Thermo Fischer Scientific, Waltham, Massachusetts) as detailed by the manufacturer.

The HUH7 cell line was used to generate tumor spheroids based in part on previously established protocols.⁷ Briefly, cell attachment was inhibited by coating culture dishes with the hydrogel poly(2-hydroxyethyl methacrylate) (polyHEMA, Sigma-Aldrich, St. Louis, Missouri). The cells were cultured under normal cell culture conditions (37°C, 5% CO₂) in a medium consisting of Dulbecco's modified eagle's medium with GlutaMAX (Gibco, Thermo Fischer Scientific, Waltham, Massachusetts) containing 10% fetal calf serum (Merck, Darmstadt, Germany) and 1% penicillin/streptomycin (General Electric, Fairfield, Connecticut) in the treated dishes. After 5 to 7 days, spheroids between 200 and 300 μ m in diameter were used for the invasion assay.

A standardized invasion protocol was established to allow comparable results. To this end, 2.5×10^4 CMFDA stained MSCs (determined using a Neubauer counting chamber) were added to a 1.5-ml reaction tube containing a single spheroid in a total volume of 50 μ l cell culture medium. The tube was then rotated horizontally at 36 rpm for 2 h at room temperature, thus ensuring an even distribution and attachment of MSC to the spheroid surface. MSCs that had not attached were washed away using three times 1-ml cell culture medium. Each spheroid-MSC preparation was incubated in a volume of 50 μ l cell culture medium for a period of 24 h in a polyHEMA coated well of a 96-well plate under normal cell culture conditions. Fixation using 4% formalin (Sigma-Aldrich, St. Louis, Missouri) for 2 h at room temperature and storage in 50% glycerol (Roth, Karlsruhe, Germany) and 0.2% propyl gallate (Sigma-Aldrich, St. Louis, Missouri) in phosphate-buffered saline at 4°C to avoid bleaching allows large numbers of spheroids to be tested in parallel.

Quantification of invasion depth requires localization of individual MSC. Widefield microscopy of whole spheroids did not provide usable information in this 3-D context. Cryosectioning allows analysis in the third dimension, but requires extensive sample preparation. To this end, "optical sectioning" via 3-D imaging techniques was used (see Fig. 1, Videos 1 and 2). As compared to two-photon laser scanning microscopy, light sheet technology allows rapid image acquisition, and therefore, lends itself well to this screening approach.

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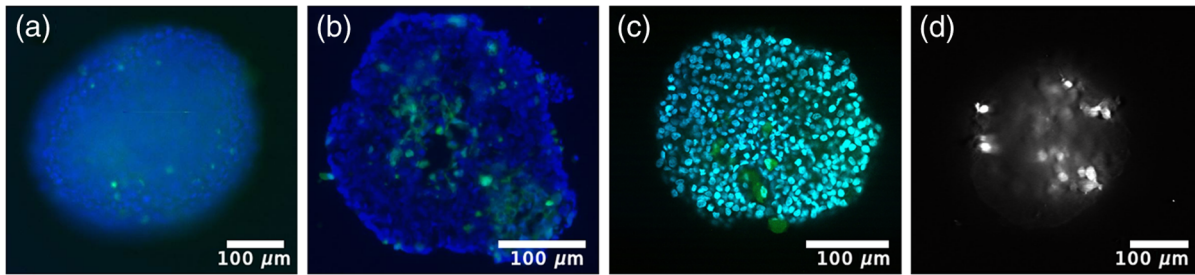


Fig. 1 Methods to image mesenchymal stem cell (MSC) invasion into tumor spheroids. Human MSCs were stained with 10 μ M (a–c) or 1 μ M (d) CellTracker Green CMFDA (Molecular probes®) prior to invasion. The cells were incubated for 24 h with HUH7 spheroids as described. Whole spheroids were fixed with 4% formalin and, in (a–c), DAPI stained. (a) and (b) show two-dimensional (2-D) images: widefield microscopy of (a) whole spheroid, and (b), a cryosection, signals of 365 nm (blue) and 470 nm (green) excitation were merged. (c) and (d) show single optical slices out of three-dimensional (3-D) stacks imaged with (c) two-photon microscopy with an excitation wavelength of 760 nm and laser power of 120 mW, signals from emission channels below (blue) and above (green) 500 nm were merged, and (d) selective plane illumination microscopy (SPIM) OpenSPIM using a 488 nm laser. Video 1: Visualization of passage three MSC invasion into tumor spheroids. Deconvolved SPIM data, animation done with the help of AMIRA Software (FEI, Dawson, Oregon). (Video 1, MPEG, 17.5 MB) [URL: <http://dx.doi.org/10.1117/1.JBO.20.4.040501.1>]. Video 2: Visualization of passage seven MSC invasion into tumor spheroids. Deconvolved SPIM data, animation done with the help of AMIRA Software (FEI, Dawson, Oregon). (Video 2, MPEG, 17.4 MB) [URL: <http://dx.doi.org/10.1117/1.JBO.20.4.040501.2>].

Light-sheet or SPIM⁸ is based on the principle of laser excitation confined to the focal plane. Specimens are illuminated by a laser light sheet perpendicular to the detection axis thereby depleting the out of focus signal to a minimum. Samples are rotated to acquire stacks from several angles prior to computation of 3-D reconstructions.

The spheroids were embedded in 1% 2-hydroxyethylagarose (Type VII, low gelling temperature, Sigma Aldrich, Missouri) mixed with fluorescent microspheres (F-XC 50 Estapor, Merck, Darmstadt, Germany; 1:4000 dilution) and aspirated into a glass capillary. Mounted on an OpenSPIM set-up, they were imaged with the solidified agarose hanging directly in phosphate-buffered saline in front of the detection lens. The SPIM application is available via open access hardware and open source software^{9,10} (detailed instructions of SPIM assembly, sample mounting, and imaging¹¹).

SPIM imaging was performed using a 488 nm laser (Cube, Coherent, Santa Clara, California; 2 mW laser power, 120 ms exposure time) and an sCMOS camera (Orca-flash 4.0 V2, Hamamatsu, Hamamatsu City, Japan) from five different angles equally spaced over 360 deg, controlled via the Fiji μ Manager plugin.^{12,13} The subsequent bead registration and fusion of images were performed using open source software on Fiji.^{14,15} To minimize data volume, images were acquired with a 2×2 binning, and for subsequent analysis down sampled by a factor of 4. For visualization, images were deconvolved without down-sampling (Videos 1 and 2).¹⁶ The resolution acquired with a 20 \times water immersion objective (NA 0.5) and a lightsheet thickness of 15 μ m full width at half maximum is 540 nm \pm 112 in x, 599 nm \pm 128 in y, and 3908 nm \pm 954 in z for raw image stacks, 1159 nm \pm 185 in x, 862 nm \pm 99 in y, and 1933 nm \pm 1089 in z for fused data, and 378 nm \pm 127 in x, 355 nm \pm 67 in y, and 978 nm \pm 58 in z after deconvolution.¹⁷

The CMFDA signals were segmented using the Fiji 3-D object counter plugin.¹⁸ The fused 32-bit grayscale images (16-bit can be alternatively chosen with the updated version of the plugin) were converted into 8-bit by linearly scaling the display range from 0 to 9000 pixel values from the original. Hence,

a segmentation threshold was typically set to 70 pixel values and the minimum size filter was set to 200 voxels (to exclude fiducial beads and artifacts).

The autofluorescent spheroid was segmented in the same manner with a threshold between 14 and 18 pixel values (tested in advance) and a minimum size of 10^5 5 voxels. Invasion depths were quantified by the measurement of distances from the center of each MSC to the border of the spheroid using the Fiji 3-D manager plugin.¹⁹ The image processing and analysis steps were conducted in a semi-automated fashion via macros developed with ImageJ.²⁰

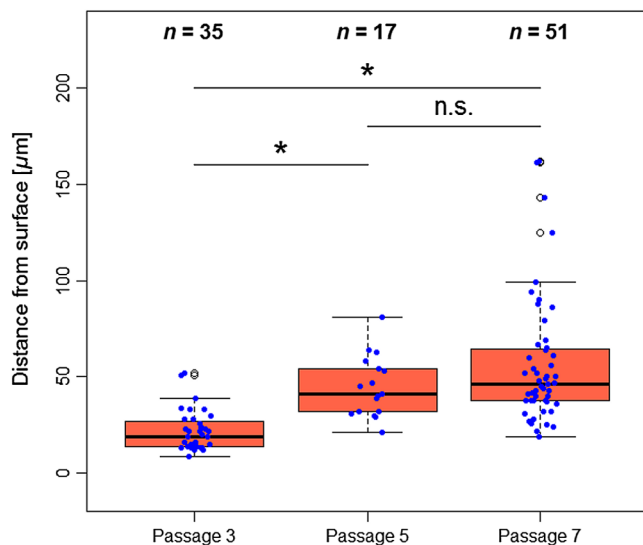


Fig. 2 Quantification of MSC invasion into tumor spheroids. The migratory distances of individual MSC from the spheroid surface are plotted. Primary MSCs from passages three, five, and seven were analyzed following an invasion time of 24 h. Three spheroids per condition were imaged, total numbers of MSC found (*n*) are indicated above. n.s.: not significant, **P* < 0.01 (Kruskal–Wallis test) (see also Fig. 1, Videos 1 and 2).

Cellular activation on plastic and components of standard culture media including factors released by MSC themselves have been shown to influence the migratory behavior of (stem) cells.²¹ As a proof of concept for the assay, the effect of expanded culture on the passaging of MSC isolated from one donor was tested. The results suggested an enhanced ability of the cells to invade the spheroid after expanded passaging. A significant increase of invasion depths was found between passages three and five. Longer passaging of up to passage seven showed no further increase (see Fig. 2). The results support the contention that limited expansion of early isolates may help to shape their tumor tropism.

To optimize MSC-based tumor therapies, one important parameter is the migratory efficiency of MSC into tumor tissue. The *in vitro* invasion assay described here provides a straightforward method for the analysis of MSC lots and pretreatment regimens on the ability of the cells to invade experimental tumors. Importantly, this approach should also be directly applicable for the investigation of other tumor infiltrating cell types, such as leukocytes, which are also under development for advanced tumor therapy.

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References

1. H. F. Dvorak, “Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing,” *N. Engl. J. Med.* **315**(26), 1650–1659 (1986).
2. K. Shinagawa et al., “Mesenchymal stem cells enhance growth and metastasis of colon cancer,” *Int. J. Cancer* **127**(10), 2323–2333 (2010).
3. J. C. von Einem et al., “TREAT-ME 1: treatment of advanced gastrointestinal cancer in a clinical phase I/II trial with genetically modified mesenchymal stem cells: 2014 ASCO annual meeting,” *J. Clin. Oncol.* **32**(suppl; abstr TPS3122[^]), 5s (2014).
4. Q. Bao et al., “Mesenchymal stem cell-based tumor-targeted gene therapy in gastrointestinal cancer,” *Stem Cells Dev.* **21**(13), 2355–2363 (2012).
5. R. Sutherland, “Cell and environment interactions in tumor microregions: the multicell spheroid model,” *Science* **240**(4849), 177–184 (1988).
6. B. Mayer et al., “Multicellular gastric cancer spheroids recapitulate growth pattern and differentiation phenotype of human gastric carcinomas,” *Gastroenterology* **121**(4), 839–852 (2001).
7. A. Ivascu and M. Kubbies, “Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis,” *J. Biomol. Screening* **11**(8), 922–932 (2006).
8. J. Huisken, “Optical sectioning deep inside live embryos by selective plane illumination microscopy,” *Science* **305**(5686), 1007–1009 (2004).
9. P. G. Pitrone et al., “OpenSPIM: an open-access light-sheet microscopy platform,” *Nat. Methods* **10**(7), 598–599 (2013).
10. E. Gualda et al., “Going ‘open’ with mesoscopy: a new dimension on multi-view imaging,” *Protoplasma* **251**(2), 363–372 (2014).
11. “OpenSPIM,” 16 August 2014, http://openspim.org/Welcome_to_the_OpenSPIM_Wiki (accessed 23 March 2015).
12. J. Schindelin et al., “Fiji: an open-source platform for biological-image analysis,” *Nat. Methods* **9**(7), 676–682 (2012).
13. A. Edelstein et al., “Computer control of microscopes using μ Manager,” *Curr. Protoc. Mol. Biol.* **92**, 14–20 (2010).
14. S. Preibisch et al., “Software for bead-based registration of selective plane illumination microscopy data,” *Nat. Methods* **7**(6), 418–419 (2010).
15. S. Preibisch et al., “Mosaicing of single plane illumination microscopy images using groupwise registration and fast content-based image fusion,” *Proc. SPIE* **6914**, 69140E (2008).
16. S. Preibisch et al., “Efficient Bayesian-based multiview deconvolution,” *Nat. Methods* **11**(6), 645–648 (2014).
17. J. Krieger, “MATLAB-script for dualview beadscans,” *DKFZ*, 25 September 2014, <http://www.dkfz.de/Macromol/quickfit/beadscan.html> (2 March 2015).
18. S. Bolte and F. P. Cordelières, “A guided tour into subcellular colocalization analysis in light microscopy,” *J. Microsc.* **224**(3), 213–232 (2006).
19. J. Ollion et al., “TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization,” *Bioinformatics* **29**(14), 1840–1841 (2013).
20. S. Rühland, D. Hörnl, and H. Harz, “Invasion assay,” 19 December 2014, http://fiji.sc/Invasion_assay (accessed 23 March 2015).
21. R. A. Boomsma, D. L. Geenen, and T. G. Hofmann, “Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis,” *PLoS One* **7**(4), e35685 (2012).