Published in final edited form as:

Curr Mol Med. 2009 November ; 9(8): 1017–1023.

Multiplexed Fluorescence Imaging of Tumor Biomarkers in Gene Expression and Protein Levels for Personalized and Predictive Medicine

Mark Q. Smith, Charles A. Staley, David A. Kooby, Toncred Styblo, William C. Wood, and Lily Yang*

Department of Surgery and Winship Cancer Institute, Emory University School of Medicine, C-4088, 1365 C Clifton Road NE, Atlanta, GA 30322, USA

Abstract

Combining ground breaking research and developments in cancer biomarkers, nanotechnology and molecular targeted medicine, a new realm of therapy is possible: personalized and predictive medicine. Developing a method to detect the overexpression of several tumor marker genes simultaneously, knowing that a single cell generally expresses more than one altered gene, should have a high predictive value for identifying cancer cells amidst the normal cellular background. Theoretically, a cancer's unique molecular profile can be used to predict its invasive and metastatic potential, its ability to evade immune surveillance, and its potential response to treatment. Fluorescent probes have been developed to detect the levels of expression of various biomarkers in tumor cells and tissues. Expression of biomarker messenger RNAs (mRNAs) or the presence of a specific mutation in an oncogene in cancer cells can be detected using molecular beacons (MBs) that only emit fluorescent signals after binding to its specific target mRNAs. Antibodies or ligands labeled with fluorophores or fluorescent quantum dots (QDs) have been successfully used to identify specific proteins expressed in cells. Furthermore, multiplex imaging using both MBs and antibodies labeled with a fluorescent probe on the same sample may provide important information correlating the level of mRNA expression and the subsequent level of protein production for a given biomarker. This technology will be useful in research investigating cancer biology, molecular imaging and molecular profiling. With the identification of biomarkers that are related to aggressive tumor types, we may be able to predict within certain patient populations who will develop invasive cancers, and what their prognosis will be given different treatment modalities, ultimately delivering medical care and treatment strategies that are specifically tailored to each individual patient, making personalized and predictive medicine a reality.

Keywords

Biomarkers; molecular beacons; molecular profiling; multiplexed imaging; nanotechnology; personalized medicine; predictive medicine; quantum dots

Approximately 1.5 million people are diagnosed with cancer this year in the United States and roughly six hundred thousand deaths due to cancer will occur. For people under the age of 85, the number of deaths due to cancer now outranks the number of deaths due to heart

disease for the first time since these statistics have been assessed [1]. Based on the scope of the problem, increasingly sensitive and specific methods to accurately diagnose, effectively treat, and potentially prevent the evolution of cancer from microscopic disease to macroscopic and metastatic disease are needed. Through developments in molecular genetics, nanotechnology and biomedicine, a new realm of therapy is possible: personalized and predictive medicine.

To make the jump from the current state of existing diagnostic and treatment modalities to the future of personalized and predictive medicine, one important step will be to integrate the science of cancer biomarkers with the world of nanotechnology. Currently, investigators are identifying mutated genes and altered products of cellular machinery (RNAs, proteins, metabolites) associated with cancer. These biomarkers may act as indicators for a defined clinical outcome [2-16]. Identifying biomarkers associated with aggressive behavior may help diagnose and possibly predict the course of a malignancy based on the patient's molecular profile; resulting in the ability to predict the invasive and metastatic potential of a cancer, its immunologic properties, and its response to treatment [17-20]. At present, an obstacle to linking a specific biomarker or set of biomarkers to a particular cancer behavior is in the lack of sufficient verifiable data. This problem exists due to the fact that human cancer tissues are substantially heterogeneous and the cancerous cells have undergone multiple genetic alterations. Cancer cells express a range of biomarkers at varying levels [21-23]. Compounding this situation the tumor cells themselves are surrounded by both normal stroma and active tumor stromal cells each expressing their own biomarkers. At present, morphologic assessment with immunohistochemical analysis remains the standard for clinical specimen analysis. Other means, such as *in situ* hybridization using fluorescent-labeled linear probes, can be used to detect gene expression in tissue sections; unfortunately, this method is inefficient and carries high background noise as free probes can emit fluorescent signals [24]. Conventional methods for pathological and molecular analysis of human cancer cells and tissues do not fully capture the features of biomarkers and use them as a means to detect cancer cells, ascertain a molecular profile of a tumor type and thereby predict the clinical outcome of cancer patients. Taking all this into consideration, if one could detect the overexpression of several tumor marker genes simultaneously, knowing that a single cell generally expresses more than one altered gene, this should have a high predictive value for identifying cancer cells. The ability to simultaneously identify multiple biomarkers within a cancer should improve our understanding of disease, and ultimately allow us to tailor therapy to a given cancer profile. Recent advances in molecular cellular imaging using novel fluorescent probes offer great opportunity to detect the level of messenger RNA (mRNA) and proteins of biomarkers simultaneously in a single cell. Using a gene specific-activated fluorescent imaging nanoprobe (molecular beacon), a relative level of a specific mRNA can be determined in intact human cancer cells.

Although the methods for detection of the level of biomarker proteins using fluorescent dye labeled antibodies have been well developed, simultaneous detection of the expression levels of several biomarkers has been a major challenge using conventional immunofluorescence labeling since limited numbers of fluorescent dye molecules can be used to label the same sample, due to a relatively wide emission peak of a fluorophore. Emerging as a new class of fluorescent probes for biomolecular and cellular imaging, quantum dots (QDs) are tiny, nanometer-scale light-emitting particles. In comparison with organic dyes, quantum dots have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and ability to simultaneously excite multiple fluorescent colors. These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by 1-2 orders of magnitude [25]. Therefore, the development of fluorescent imaging QD probes makes it possible to detect and quantify the protein level of several biomarkers as

well as post-translational modifications simultaneously within the same cell. In this review, we will discuss in detail the current development of two fluorescent nanoimaging probes and their potential applications in detecting and phenotyping cancer cells within clinical samples.

MOLECULAR BEACONS

A molecular beacon (MB) is a sequence of oligonucleotides that has a fluorophore attached to one end and a quencher at the opposite end. Under native conditions, the MB self-hybridizes due to the complementary sequences at the 5' and 3'-ends, and assumes a stem-loop structure. This brings the fluorophore and the quencher into close proximity; as a result, in the absence of specific target molecules, the fluorophore is quenched and a fluorescent signal is not emitted. When the molecular beacon hybridizes to its specific target sequence, the stem is broken, separating the fluorophore from the quencher, allowing the fluorophore to emit its signal [26-28] Fig. (1A). Conditions between the loop of the molecular beacon and its complementary target sequence that allow hybridization are very rigorous. The matching specificity is so high that a difference as small as one base pair between the molecular beacon and a possible target will prevent hybridization [27-29]. This technology can be used to examine several *in vitro* processes: identifying biomarkers associated with specific cancers, detection of DNA mutations, real-time quantification of PCR products, and monitoring protein-DNA interactions [28-30]. Additionally, molecular beacon probes have been used to identify intracellular mRNA molecules within intact cells [31-35]. The feasibility of detecting intracellular mRNA has been extensively examined and has successfully visualized mRNA molecules in human and animal cell lines [31-36]. Further research has determined that molecular beacons can be used as a highly sensitive method for detecting mRNA within cells [31]. Results of our study have successfully identified the known tumor biomarkers Survivin and Cyclin D1 in breast cancer cells using Survivin or Cyclin D1 MB Fig. (1B), and mutant K-ras mRNA in pancreatic cancer cells using K-ras MBs specific for point mutations located at K-ras codon 12 [24, 36]. Moreover, changes in the level of mRNA expression of a specific gene can be monitored in real-time in living cells to determine the effect of biological factors or chemotherapeutic agents on a specific molecular target [24]. Using this molecular beacon technology, one could examine a single cell for the presence of multiple tumor biomarkers Fig. (1). This is possible since molecular beacons have high targeting specificity and each molecular beacon can be labeled with a different fluorescent dye molecule. Due to the fact that unbound molecular beacons do not emit fluorescent signals and each molecular beacon has only one fluorophore, the intensity level of fluorescence generated by the hybridization of a molecular beacon to its target mRNA should therefore indicate a true expression level of mRNA in cells [24].

QUANTUM DOTS

QDs are part of a new group of fluorescent probes for biomolecular and cellular imaging [37-44]. Comparing the properties of QDs to those of traditional organic dyes and fluorescent proteins, QDs have highly advantageous and distinctive optical and electronic characteristics. QDs are much brighter than organic dyes due to their larger molar extinction coefficients. Additionally, the emission wavelength of QDs is determined by their diameter. Smaller QDs (2 nm in diameter) produce emission wavelengths in the blue end of the electromagnetic spectrum while larger QDs (7 nm in diameter) produce emission wavelengths in the red end of the electromagnetic spectrum [45]. Researchers are currently developing QDs that have the ability to emit light in the near infrared spectrum (650 nm to 950 nm), which significantly reduces background fluorescence and generates higher resolution, allowing for a more accurate quantification of nanoprobe [46]. Another valuable property of QDs for imaging is the wide QD Stokes shift. Dependent upon the excitation

wavelength, the difference between the excitation peak wavelength and emission peak wavelength can be as large as 300-400 nm. This large Stokes shift in combination with the broadband absorption and sharp emission peaks of QDs make it possible to perform multiplexed imaging applications where one light source is used to simultaneously excite multicolor QDs [47]. QDs also possess long-term photostability in comparison to traditional fluorescent probes and do not experience photobleaching with prolonged exposure to light. Cumulatively, these characteristics allow for the qualification and quantification of biomarkers, whereas traditional immunohistochemistry is only qualitative. Additionally, using QDs, one could continuously study the dynamics of cellular processes over time, such as tracking cell migration, differentiation, and metastasis [48]. By conjugating antibodies, targeting ligands, or short peptides to the surface of QDs, one can actively seek out and identify cancer cells or any cellular target of interest with both high sensitivity and specificity. QDs can be functionalized to bind to biomarkers in several different ways. Antibody fragments can be conjugated to QDs by a disulphide reduction and sulfhydryl-amine coupling [48]. The primary amines of intact antibodies can be covalently linked to carboxyl groups using EDAC as a catalyst Fig. (2A). Ni-NTA modified QDs can bind histidine-tagged peptides or antibodies. Non-covalent linkage can be performed between streptavidin-coated QDs and biotinylated antibodies [48] Fig. (2A). Starting with initial work in using non-targeted QDs to map sentinel lymph nodes [49], to targeting tumor vasculature [50], and into the current development of multifunctional probes for both targeting and imaging tumors in living animals [42], the field is continually advancing with ever-expanding potential. Evidence of this is shown in the ability to target and monitor cellular receptors and intracellular trafficking using targeted QDs [43]. Multiple markers can be visualized on one cell for *in vitro* multiplexed imaging; this will allow researchers to generate a molecular profile of the biomarkers expressed in human cancer cells and tissues. One report details the examination and profiling of five receptors in breast cancer (Her2, ER, PR, EGFR, mTOR) using multicolor QDs [51]. Multiple studies investigating QD labeling have been performed on both live and fixed cells, as well as freshly harvested tissues [52-56]. And now protocols exist for utilizing this technology for probing archived formalin-fixed paraffin-embedded tissues [48]. This will allow investigators to correlate a known clinical history and outcome with the associated biomarker profile of the cancer tissue of interest.

THE ADVANTAGE OF COMBINING MB AND QDS TO DETECT mRNA AND PROTEIN LEVELS

Multiplex imaging using both molecular beacons and quantum dots on the same sample has the potential to bring to light a wealth of useable information. Although identifying tumor marker genes has been a boon to medical science, there is the unfortunate issue that most tumor marker genes are expressed by both cancerous and normal cells. At the molecular level, the basic difference between a normal cell and a cancer cell is defined by the rate at which these particular genes are expressed. Further complicating the matter of diagnosis is that in assessing clinical samples, only a small portion of the sample is composed of abnormal cells. In comparing the molecular beacon method of identifying target mRNA to the conventional RT-PCR method of amplifying the expression of tumor marker genes from isolated total RNA; molecular beacons are superior in many ways. For example, using molecular beacons obviates the inherent difficulty that exists in detecting the differences in the level of gene expression in a few cancer cells over the normal background that is prevalent in RT-PCR [57]. Therefore, there is a clear advantage in using direct fluorescence imaging to identify individual cells that are over-expressing tumor marker genes as a means to diagnose cancer. Performing multiplexed imaging of clinical samples with both molecular beacons and QDs will allow researchers to map out the total biomarker population in a

cellular sample and obtain a data set from the heterogeneous cell population of a tumor. These nanoprobe can also shed light on the intracellular pathways that cancer cells utilize to dodge apoptosis. By looking at post-translational modifications (i.e. phosphorylation/de-phosphorylation) and changes in protein levels due to increased degradation during treatment, it can be shown if the treatment is working as expected. For example, activation of the EGFR signaling pathway plays an important role in tumor cell proliferation, survival and invasion. However, detection of total level of EGFR does not reflect the activity of the EGFR pathway since the level of phosphorylated EGFR, p-EGFR, correlates with the activity of the receptor activation. We have previously reported that activation of EGFR signaling increases the transcription of an inhibitor of apoptosis family of protein, survivin [58]. Using multiplexed fluorescence cell imaging, we are able to detect the levels of survivin mRNA using survivin MB and survivin protein level using QDs conjugated with SURVIVIN antibodies Fig. (2B). Importantly, the level and activity of upstream activator for survivin, EGFR or p-EGFR, can also be determined using corresponding antibody conjugated QDs that emit at different wavelengths Fig. (2B). Therefore, using both molecular beacons and antibody (or peptide) coated QDs to perform multiplexing of the gene and protein expression of key biomarkers as a means of profiling these markers has potential for determining the changes in breast cancer cells obtained from biopsy samples before, during and after treatment of various therapeutic agents. In so doing, physicians could potentially select patients for molecular targeted therapy and for monitoring their therapeutic response to treatment. Combining information from the molecular profiles of groups of patients, researchers can generate data sets for each type of cancer and potentially predict a patient's clinical course by correlating a patient's particular multiplexed molecular beacon and QDs profile against the known data set for that cancer type. As a result, the information from a patient's particular biomarker profile could then be used to predict the type, grade, and stage of cancer present, its aggressiveness and potential response to chemotherapeutic agents and/or radiation. It may even be possible that with the identification of pre-invasive or metastatic biomarkers, we can predict within certain patient populations who will develop invasive or metastatic cancers, and what their prognosis will be, therapeutic strategies that are mostly suitable for individual patients will be used to achieve effective personalized therapy.

Acknowledgments

Dr. Yang's research Laboratory is supported by the Idea Award of the Breast Cancer Research Program of the Department of Defense (BC021952), Emory-Georgia Tech Nanotechnology Center for Personalized and Predictive Oncology of NIH NCI Center of Cancer Nanotechnology Excellence (CCNE, U54 CA119338-01), NIH R01-CA95643, and the Nancy Panoz Chair of Surgery in Cancer Research.

REFERENCES

- [1]. United States Cancer Statistics. Centers for Disease Control and Prevention (CDC); <http://www.cdc.gov/cancer/npcr.uscs>
- [2]. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES. Science. 1999; 286:531–537. [PubMed: 10521349]
- [3]. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Matt Van de Rijn M.V.d. Waltham M, Pergamenschikov A, Lee JCF, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO. Nat. Genet. 2000; 24:227–35. [PubMed: 10700174]
- [4]. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JJ, Yang L, Marti GE, Moore T, Hudson J, Lu L Jr. Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, D. Botstein D, Brown PO, Staudt LM. Nature. 2000; 403:503–11. [PubMed: 10676951]

- [5]. Perou CM, Sørli T, Eisen MB, Rijn M.v.d. Jeffrey SS, Rees CA, Pollack JR, Ross, Johnsen H, Akslen LA, Fluge Ø, Pergamenschikov A., Williams, C. Zhu SX, Lønning PE, Børresen-Dale A-L, Brown PO, Botstein D. *Nature*. 2000; 406:747–52. [PubMed: 10963602]
- [6]. Bittner M, Meltzer P, Chen Y, Jiang Y, Settor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A., Sampas, N. Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V, Hayward N, Trent J. *Nature*. 2000; 406:536–40. [PubMed: 10952317]
- [7]. Dhanasekaran SM, Barrette TR, Ghosh D., Shah, R. Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. *Nature*. 2001; 412:822–26. [PubMed: 11518967]
- [8]. Kuefer R, Varambally S, Zhou M, Lucas PC, Loeffler M, Wolter H, Mattfeldt T, Hautmann RE, Gschwend JE, Barrette TR, Dunn RL, Chinnaiyan AM, Rubin MA. *Am. J. Pathol.* 2002; 161:841–48. [PubMed: 12213712]
- [9]. Rhodes DR, Barrette TR, Rubin MA, Ghosh D, Chinnaiyan AM. *Cancer Res.* 2002; 62:4427–4433. [PubMed: 12154050]
- [10]. Rhodes DR, Sanda MG, Otte AP, Chinnaiyan AM, Rubin MA. *J. Natl. Cancer Inst.* 2003; 95:661–68. [PubMed: 12734317]
- [11]. Rubin MA. *J. Pathol.* 2001; 195:80–86. [PubMed: 11568894]
- [12]. Rubin MA, Strawderman MDR, Pienta K. *Am. J. Surg. Pathol.* 2002; 26:312–19. [PubMed: 11859202]
- [13]. Rubin MA, Zhou M, Dhanasekaran SM, Varambally S. *JAMA.* 2002; 287:1662–70. [PubMed: 11926890]
- [14]. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, A B, Otte AP, Rubin MA, Chinnaiyan AM. *Nature*. 2002; 419:624–29. [PubMed: 12374981]
- [15]. Nelson WG, De Marzo AM, Isaacs WB. *N. Engl. J. Med.* 2003; 349:366–81. [PubMed: 12878745]
- [16]. Gonzalgo ML, Pavlovich CP, Lee SM, Nelson WG. *Clin. Cancer Res.* 2003; 9:2673–77. [PubMed: 12855646]
- [17]. Weinshilboum R, Wang LW. *Nat. Rev. Drug Discov.* 2004; 3:739–48. [PubMed: 15340384]
- [18]. Evans WE, Reiling MV. *Nature*. 2004; 429:464–68. [PubMed: 15164072]
- [19]. Lynch TJ, Daphne MD, Bell W. Sordella, R. Gurubhagavatula S, Ross MD, Okimoto A, Brian BS, Brannigan W., Patricia, B.A. Harris L, Sara MS, Haserlat M, Jeffrey B.A., Supko, G. Frank G, Haluska MD, David N, Louis MD, David C, Christiani MD, Settleman J, Haber DA. *N. Engl. J. Med.* 2004; 350:2129–39. [PubMed: 15118073]
- [20]. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. *Science*. 2004; 304:1497–500. [PubMed: 15118125]
- [21]. Michener CM, Ardekani AM, Petricoin EF, Liotta LA, Kohn EC. *Cancer Detect. Prev.* 2002; 26:249–55. [PubMed: 12430629]
- [22]. Dickson, RB.; Lippman, ME. Cancer of the breast. Molecular biology of breast cancer. In: DeVita, VTJ.; Helman, S.; Rosenberg, SA., editors. Principles and Practice of Oncology. Lippincott Williams & Wilkins; Philadelphia: 2001. p. 1633-45.
- [23]. Nathanson KL, Wooster R, Weber BL, Nathanson KN. *Nat. Med.* 2001; 7:552–6. [PubMed: 11329055]
- [24]. Peng X-H, Cao Z-H, Xia J-T, Carlson GW, Lewis MM, William C, Wood LY. *Cancer Res.* 2005; 65:1909–17. [PubMed: 15753390]
- [25]. Gao XH, Cui YY, Levenson RM, Chung LWK, Nie SM. *Nat. Biotechnol.* 2004; 22:969–76. [PubMed: 15258594]
- [26]. Tyagi S, Kramer FR. *Nat. Biotechnol.* 1996; 14:303–8. [PubMed: 9630890]
- [27]. Bonnet G, Tyagi S, Libchaber A, Kramer FR. *Proc. Natl. Acad. Sci. USA.* 1999; 96:6171–6. [PubMed: 10339560]
- [28]. Tan W, Fang X, Li J, Liu X. *Chemistry*. 2000; 6:1107–11. [PubMed: 10785794]
- [29]. Tyagi S, Bratu DP, Kramer FR. *Nat. Biotechnol.* 1998; 16:49–53. [PubMed: 9447593]

- [30]. Heyduk T, Heyduk E. *Nat. Biotechnol.* 2002; 20:171–6. [PubMed: 11821863]
- [31]. Sokol DL, Zhang X, Lu P, Gewirtz AM. *Proc. Natl. Acad. Sci. USA.* 1998; 95:11538–43. [PubMed: 9751701]
- [32]. Dirks RW, Molenaar C, Tanke HJ. *Histochem. Cell Biol.* 2001; 115:3–11. [PubMed: 11219605]
- [33]. Fang X, Mi Y, Li JJ, Beck T, Schuster S, Tan W. *Cell Biochem. Biophys.* 2002; 37:71–81. [PubMed: 12482132]
- [34]. Bratu DP, Cha BJ, Mhlanga MM, Kramer FR, Tyagi S. *Proc. Natl. Acad. Sci. USA.* 2003; 100:13308–13. [PubMed: 14583593]
- [35]. Shah R, El-Deiry WS. *Cancer Biol. Ther.* 2004; 3:871–5.
- [36]. Yang L, Cao Z, Lin Y, Wood WC, Staley CA. *Cancer Biol. Ther.* 2005; 4:561–70. [PubMed: 15917666]
- [37]. Alivisatos P. *Nat. Biotechnol.* 2004; 22:47–52. [PubMed: 14704706]
- [38]. Alivisatos AP. *Science.* 1996; 271:933–37.
- [39]. Alivisatos AP, Gu WW, Larabell C. *Annu. Rev. Biomed. Eng.* 2005; 7:55–76. [PubMed: 16004566]
- [40]. Pinaud F, Michalet X, Bentolila LA, Tsay JM, Doose S, Li JJ, Iyer G, Weiss S. *Biomaterials.* 2006; 27:1679–87. [PubMed: 16318871]
- [41]. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. *Science.* 2005; 307:538–44. [PubMed: 15681376]
- [42]. Gao XH, Yang LL, Petros JA, Marshal FF, Simons JW, Nie SM. *Curr. Opin. Biotechnol.* 2005; 16:63–72. [PubMed: 15722017]
- [43]. Smith AM, Gao X, Nie S. *Photochem. Photobiol.* 2004; 80:377–85. [PubMed: 15623319]
- [44]. Chan WCW, Maxwell DJ, Gao XH, Bailey RE, Han MY, Nie SM. *Curr. Opin. Biotechnol.* 2002; 13:40–46. [PubMed: 11849956]
- [45]. Yu, W.W., Qu, L.H.; Guo, W.Z.; Peng, X.G. *Chem. Mater.* 2003; 15:2854–60.
- [46]. Kim SW, Zimmer JP, Ohnishi S, Tracy JB, Frangioni JV, Bawendi MG. *J. Am. Chem. Soc.* 2005; 127:10526–32. [PubMed: 16045339]
- [47]. Nie S, Xing Y, Kim GJ, Simons JW. *Annu. Rev. Biomed. Eng.* 2007; 9:12.1–32.
- [48]. Xing Y, Chaudry Q, Shen C, Kong KY, Zhau HE, Chung LW, Petros JA, O'Regan RM, Yezhelyev MV, Simons JW, Wang MD, Nie S. *Nat. Protocols.* 2007; 2:1152–65.
- [49]. Kim S, Lim YT, Soltész EG, De Grand AM, Lee J, Nakayama A, Parker JA, Mihaljevic T, Laurence RG, Dor DM, Cohn LH, Bawendi MG, Frangioni JV. *Nat. Biotechnol.* 2003; 22:93–97. [PubMed: 14661026]
- [50]. Akerman ME, Chan WCW, Laakkonen P, Bhatia SN, Ruoslahti E. *Proc. Natl. Acad. Sci., USA.* 2002; 99:12617–21. [PubMed: 12235356]
- [51]. Maksym V, Yezhelyev, AI-Hajj A, Morris C, Marcus AI, Liu T, Lewis M, Cohen C, Zrazhevskiy P, Jonathan W, Simons, Rogatko A, Nie S, Gao X, O'Regan RM. *Adv. Mater.* 2007; 19:3146–51.
- [52]. Ness JM, Akhtar RS, Latham CB, Roth KA. *J. Histochem. Cytochem.* 2003; 51:981–87. [PubMed: 12871979]
- [53]. Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. *Nat. Biotechnol.* 2004; 22:198–203. [PubMed: 14704683]
- [54]. Xingyong, W.u.; Liu, H.; Liu, J.; Haley, KN.; Treadway, JA.; Larson, JP.; Ge, N.; Peale, V.; Bruchez, MP. *Nat. Biotechnol.* 2003; 21:41–46. [PubMed: 12459735]
- [55]. Tokumasu F, Dvorak JJ. *Microsc.* 2003; 211:256–61.
- [56]. Ferrara DE, Weiss D, Carnell PH, Vito RP, Vega D, Gao X, Nie S, Taylor WR. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2006; 290:R114–23.
- [57]. Srinivas PR, Verma M, Zhao Y, Srivastava S. *Clin. Chem.* 2002; 48:1160–69. [PubMed: 12142368]
- [58]. Peng X, Karna P, Cao Z, Jiang B, Zhou M, Yang L. *J. Bio. Chem.* 2006; 36:25903–14. [PubMed: 16847054]

A. Schematic illustration of detection of a mRNA using a molecular beacon **B. Simultaneous detection of the level of tumor marker gene expression using MBs with different fluorescence dyes** **Fig. (1). Detection of the level of tumor marker gene expression using molecular beacons (MB)**

A. A MB is an oligonucleotide probe designed to form a stem-loop structure and dual labeled with a fluorescent dye and a quencher molecule. The probes contain specific sequences complementary to the genes of interests. In the absence of the target, the stem brings the fluorophore and quencher molecules together, which prevents emission of a fluorescent signal. Once the MB hybridizes to the target mRNA, the stem is broken, separating the fluorophore and the quencher, allowing the fluorescent signal to be emitted. The fluorescence correlates with the level of gene expression.

B. Survivin and cyclin D1 are biomarkers for breast cancer. Simultaneous delivery of MBs specific for survivin or cyclin D1 mRNA into cells produces green (survivin) or red (cyclin D1) fluorescence signal in breast cancer (MDA-MB-231 and MCF-7) but not in normal breast mammary epithelial cells (MCF-10A).

Fig. (2). Simultaneous detection of biomarker gene expression and protein levels using MB and QD probes

A. Methods for production of biomarker targeted QD probes. Antibodies are covalently linked to carboxyl groups on the surface coating of QDs *via* primary amines of the antibody (Method 1) or biotinylated antibodies non-covalently bind to streptavidin coated QDs (Method 2).

B. Multiplexed fluorescence cell imaging of gene expression and protein in cancer cells. Human breast cancer cell line, MCF-10DCIS, was cultured in chamber slides and then fixed with ice-cold acetone. The slide was incubated with survivin MB and then with QD620-survivin antibody (Ab), QD-580-EGFR Ab and QD-600 p-EGFR Ab. The cells were examined under a fluorescence microscope and fluorescence images were taken using a multi-spectral imaging system (CRI Inc.). Fluorescent colors shown are assigned for MB and QDs using the imaging system.