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Multi-color *in vivo* targeted imaging to guide real-time surgery of HER2-positive micrometastases in a two tumor coincident model of ovarian cancer

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Summary

One of the primary goals of oncologic molecular imaging is to accurately identify and characterize malignant tissues *in vivo*. Currently, molecular imaging relies on targeting a single molecule that while over-expressed in malignancy, is often also expressed at lower levels in normal tissue, resulting in reduced tumor to background ratios (TBR). One approach to increasing the specificity of molecular imaging in cancer is to employ multiple probes each with distinct fluorescence to target several surface antigens simultaneously, in order to identify tissue expression profiles, rather than relying on the expression of a single target. This next step forward in molecular imaging will rely on characterization of tissue based on fluorescence and therefore will require the ability to simultaneously identify several optical probes each attached to different targeting ligands. We created a novel “coincident” ovarian cancer mouse model by co-injecting each animal with two distinct cell lines, HER2+/RFP- SKOV3 and HER2-/RFP+ SHIN3-RFP, in order to establish a model of disease in which animals simultaneously bore tumors with two distinct phenotypes (HER2+/RFP+, HER2-/RFP+) which could be utilized for multicolor imaging. The HER2 receptor of the SKOV3 cell line was targeted with trastuzumab-rhodamine green conjugate to create green tumor implants, while the RFP plasmid of the SHIN3 cells created red tumor implants. We demonstrate that real time *in vivo* multicolor imaging is feasible and that fluorescence characteristics can then serve to guide the surgical removal of disease.

Introduction

Optical imaging represents a potentially powerful tool for the diagnosis and treatment of human disease. Cancer-specific optical probes can effectively target malignant lesions to aid in the localization of disease *in vivo* 1· 2. Optical probes can also convey information regarding the molecular nature of disease, providing valuable clinical insight about prognosis and responsiveness to therapeutics 3. Numerous studies targeting a variety of receptor types have shown that optical imaging is an effective method of characterizing cancers based on their cell-surface receptor expression 4· 5. For example, the expression of epidermal growth factor receptor 2 (EGFR-2 or HER2) is known to affect the prognosis of breast cancer patients and is the marker for antibody-directed therapy with trastuzumab, a

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monoclonal antibody directed against HER2 6-8. Optical imaging targeting HER2 can accurately detect the presence of the receptor 9.

However, targeting a single marker of cancer has limitations. A hallmark of human cancers is heterogeneity. Just as immunohistochemistry employs an array of stains to characterize tumors, the accuracy of tissue characterization with optical probes can be improved by demonstrating an expression profile of the tumor rather than relying on the expression of a single receptor. Ultimately, *in vivo* multicolor tissue characterization relies on 1) the identification of multiple targets in the same tumor, 2) target-specific optical probes with distinct fluorescent properties, and 3) effective real-time multicolor optical cameras that permit accurate unmixing of different fluorescent probes *in vivo*.

In order to determine the feasibility of real time multi-color imaging we created a unique animal model of peritoneal ovarian carcinomatosis, which permitted multicolor imaging of tumor implants. The model was established by co-injecting each animal with two distinct cell lines, HER2+/RFP- SKOV3 and HER2-/RFP+ SHIN3-RFP, in order to establish a model of disease in which animals simultaneously bore tumors with two distinct phenotypes (HER2+/RFP+, HER2-/RFP+) which could be utilized for multicolor imaging. The HER2 receptor of the SKOV3 cell line was targeted with trastuzumab-rhodamine green conjugate to create green tumor implants, while the RFP plasmid of the SHIN3 cells created red tumor implants. Using this model, we then evaluated the ability of optical imaging to discriminate between two colors during *in vivo* evaluation in a coincident ovarian tumor model of ovarian cancer. Real-time optical imaging was then used to guide the removal of mesenteric tumor implants to evaluate the clinical applicability of this technology.

Materials and Methods

Cell culture

Two established epithelial ovarian cancer cell lines were used for intraperitoneal ovarian cancer mouse models: SHIN3 10, a very low HER2expressing cell line and SKOV3, a cell line known to over-express the HER2 receptor 11. SHIN3 cells were transfected with a red fluorescent protein (RFP) expressing plasmid to create a red fluorescent phenotype tumor, as previously described 12. The cell lines were grown in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA), 0.03% L-glutamine at 37 °C, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂.

Synthesis of trastuzumab-conjugated rhodamine green

Trastuzumab, (Herceptin® Genentech, South San Francisco, CA), an FDA-approved humanized anti-HER-2 antibody, which has a complimentary determination region (CDR) against HER-2 grafted on a human IgG₁ framework, was purchased from Genentech Inc. At room temperature, 1 mg (6.85 nmol) of trastuzumab in Na₂HPO₄ was incubated with 68.5 nmol of Rhodamine Green (RhodG), at pH 8.5 for 15 minutes at room temperature. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare, Piscataway, NJ). Trastuzumab-RhodG was kept at 4°C in the refrigerator as stock solutions. The protein concentrations of trastuzumab-RhodG samples were determined with Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL) by measuring the absorption at 595 nm with a UV-Vis system (8453 Value UV-Visible Value System; Agilent Technologies, Santa Clara, CA) using standard solutions of known concentrations of trastuzumab (200 µg/ml). The concentration of RhodG was then measured by absorption at 503 nm with the UV-Vis system to confirm the number of fluorophore molecules conjugated with each

trastuzumab molecule. The number of fluorophore molecules per trastuzumab was adjusted to approximately 3.

Flow cytometry analysis

Flow cytometry analyses were performed to assess the binding of trastuzumab-RhodG to SHIN3 and SKOV3 cells and the expression of RFP in SHIN3-RFP. The cells (SHIN3, SKOV3, or SHIN3-RFP (each at 2×10^5)) were plated on a 6-chamber culture well and incubated for 16 h. Trastuzumab-RhodG was added to the medium (30 $\mu\text{g}/\text{mL}$) of SHIN3 and SKOV3 cells, and the cells were incubated for additional 8 hours. After incubation, all cells were trypsinized and washed twice with PBS, and then flow cytometry was performed with FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ). A 488nm argon ion laser was employed for excitation, and 530/30 nm band-pass filter were used for trastuzumab-RhodG emission light collection, and 585/42 nm band-pass filter for RFP. Finally, all data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Verification of differential fluorescence between SHIN3-RFP and trastuzumab-RhodG targeted SKOV3

To verify that SHIN3-RFP and trastuzumab-RhodG targeted SKOV3 cells, possessed distinct red and green fluorescence, respectively, fluorescence microscopy was conducted on cells from a cell culture containing both cell types after incubation with trastuzumab-RhodG for 1 and 8 h. SHIN3-RFP and SKOV3 cells (1×10^4) were plated on a cover glass bottom culture well and incubated for 16 h, after which trastuzumab-RhodG (30 $\mu\text{g}/\text{mL}$) was added to the medium, and the cells were incubated with optically labeled antibody for either 1h or 8h. After the respective incubation period, cells were washed five times with PBS and fluorescence microscopy was performed using an Olympus BX61 microscope (Olympus America Inc., Melville, NY). For the blue light filter, a band pass filter from 470 to 490 nm and a band pass filter from 515 to 550 nm were used for excitation and emission light, respectively; for the green light filter, the values were from 530 to 585 nm and from 605 to 680 nm, respectively. In the preliminary experiments, we verified that light from RhodG could be acquired with this filter set, while it was not identified with the green filter set. The opposite was the case for RFP. Transmitted light differential interference contrast (DIC) images were also acquired.

Coincident tumor model

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee. The intraperitoneal tumor implants were established by intraperitoneal (i.p.) injection of 2×10^6 cells suspended in 200 μL of PBS in female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). To establish tumors in which both cell types were coincident, each mouse was treated with i.p injection of SKOV3 cells and 7 days later, the same mice were treated with an i.p. injection of SHIN3-RFP cells. Experiments with dual-injected tumor-bearing mice were performed at 30 days after injection of SKOV3 cells.

Verification of the coincident tumor model

Macroscopic *in situ* multispectral fluorescence imaging was used to confirm the presence of both SHIN3-RFP and SKOV3 derived peritoneal tumors within the mesentery of dual-injection treated mice (n=5). 24 h prior to imaging experiments, mice received an intraperitoneal (i.p.) injection of a 300- μL solution containing 50 μg of trastuzumab-RhodG diluted in PBS. The dosing strategy and decision to conduct imaging experiments at 24 h were based on extrapolation from previous work^{7, 9}. Prior to imaging, mice were sacrificed

individually with carbon dioxide. Immediately after sacrifice, the abdominal cavity was exposed and loops of bowel were spread on non-fluorescent black plates. Multispectral fluorescence images were obtained of tumor nodules within the mesentery of bowel loops using the Maestro In-Vivo Imaging System (CRi, Inc., Woburn, MA) with multi-excitation acquisition⁷. A band-pass filter from 445 to 490 nm and a long-pass filter over 515 nm as well as a band-pass filter from 503 to 555 nm and a long-pass filter over 580 nm were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10 nm increments from 500 to 800 nm, while the camera captured images at each wavelength interval with constant exposure time. Spectral fluorescence images consisting of spectra due to autofluorescence, trastuzumab-RhodG conjugate, and RFP were obtained and then were “unmixed” on the basis of their spectral pattern using commercial software (*Maestro* software, CRi, Inc., Woburn, MA).

Following the macroscopic multispectral fluorescence imaging analysis, fluorescence microscopy was used to verify the presence of both SHIN3-RFP and SKOV3 derived peritoneal tumors within the mesentery of dual-injection treated mice. 24 h prior to fluorescence microscopy experiments, mice were treated with an intraperitoneal (i.p.) injection of a 300- μ L solution containing 50 μ g of trastuzumab-RhodG diluted in PBS. Microscopic tumor implants from dual-injected mice were imaged using fluorescence microscopy with an Olympus BX61 microscope (Olympus America Inc., Melville, NY). For the blue light filter, a band pass filter from 470 to 490 nm and a band pass filter from 515 to 550 nm were used for excitation and emission light, respectively; for the green light filter, the values were from 530 to 585 nm and from 605 to 680 nm, respectively. In the preliminary experiments, we had verified that light from RhodG could be acquired with the blue filter settings, but was not identified with green filter settings, and vice versa for RFP (data not shown). Transmitted light DIC images were also acquired.

***In situ* real-time tissue sampling**

After the coincident tumor model was established, image-guided tumor resection simulating surgical debulking was evaluated by removing mesenteric tumor implants. A real-time color optical imaging system, FluorVivo (INDEC Biosystems, Santa Clara, CA) was employed. Green fluorescence (trastuzumab-RhodG) was arbitrarily defined as the target tissue while red fluorescence (RFP) served as a control marker. Nodules were categorized into two groups, those demonstrating any demonstrating green (with or without red fluorescence) and those demonstrating only red fluorescence and lacking green fluorescence. Nodules, placed on a black plate according to categorization. Imaging of excised tumor nodules was performed to compare real-time multicolor optical imaging to conventional multispectral imaging with the Maestro imaging system (CRi).

***In vivo* real-time fluorescence-guided tissue biopsy**

Real time image guided tumor resections were conducted in live mice. For these *in vivo* experiments, mice (n=5) were anesthetized with inhaled 5% isoflurane and the abdominal cavity was exposed to reveal intraperitoneal tumor implants. Real-time fluorescence guided tissue resection experiments were conducted using the FluorVivo system. As with the *in situ* experiments, green fluorescence was defined as the target marker and red was defined as a control marker. All removed tumor nodules were categorized and evaluated in the same fashion as the *in situ* experiments.

Histologic verification of tumor implants

All tumor nodules removed during *in situ* and *in vivo* experiments were fixed with 10% formalin, stained with Hematoxylin-Eosin (H&E) staining, and evaluated to verify the presence of epithelial ovarian carcinoma.

Image Data Analysis

Multispectral imaging of excised tumor nodules was obtained by creating wavelength-specific images for RFP fluorescence and trastuzumab-RhodG using commercial unmixing algorithms (Nuance, CRi, Woburn, MA). Sensitivity and specificity of real-time multicolor tumor nodule characterization were calculated using unmixed images. Multispectral images corresponding to trastuzumab-RhodG were used to calculate true positive, true negative, false positive, and false negative nodules. Due to naturally occurring autofluorescence of mouse tissue that emits at green wavelengths, Image J software (<http://rsb.info.nih.gov/ij/>) was used to measure the maximum signal intensity of nodules and those demonstrating a maximum signal intensity greater than 20 arbitrary units above background. Multispectral images unmixed for RFP were used to determine the presence of red fluorescence. True positive nodules were defined as those demonstrating either green or combined green and red fluorescence on real-time and spectral imaging. False negative nodules were those in which spectral imaging data did not agree with the real-time categorization of the nodules as having green or combined green and red fluorescence. True negative nodules were defined as nodules with only red fluorescence confirmed on both real time and spectral imaging. False positive nodules were defined as nodules possessing green fluorescence on real time with spectral imaging demonstrating no apparent green fluorescence.

Results

Specific fluorescence from trastuzumab-RhodG-targeted SKOV3 and stably RFP-expressing SHIN3-RFP cells

Flow cytometry was performed to assess the specificity of green signal HER2+ SKOV3 cells, and the specificity of red signal for SHIN3-RFP. After incubation with trastuzumab-RhodG, SKOV3 cells showed a significant green fluorescence signal, while SHIN3 cells showed minimal green signal (Figure 1A). Conversely, SHIN3-RFP cells showed significant red fluorescence as compared with parent SHIN3 control cells, indicating that the majority of SHIN3-RFP cells yielded strong red fluorescence.

Verification of differential fluorescence between SHIN3-RFP and trastuzumab-RhodG targeted SKOV3

Fluorescence imaging data acquired using filter sets specific for RhodG and RFP demonstrated the presence of two cell populations, one with red fluorescence and a second with green fluorescence at 1 and 8 h after incubation with trastuzumab-RhodG (Figure 1B).

Verification of the coincident tumor model

Fluorescence microscopy verified the presence of both SHIN3-RFP and SKOV3 derived peritoneal tumors within the mesentery of dual-injected mice. Fluorescence microscopy permitted visualization of red fluorescence signal from the SHIN3-RFP tumors as well as green fluorescence from trastuzumab-RhodG binding to HER2-overexpressed SKOV3 tumor implants within the mesentery of individual mice, therefore confirming the ability of the dual injection method to establish a coincident tumor model (Figure 2).

Spectral Fluorescence Imaging

Multispectral fluorescence imaging verified the presence of both SHIN3-RFP and SKOV3 derived peritoneal tumors within the mesentery of dual-injected mice treated with i.p. trastuzumab-RhodG (Figure 2, 3a) prior to *in situ* tumor removal experiments (supplemental video 1). Spectral fluorescence images of mesenteric tumor nodules were obtained and then unmixed on the basis of their spectral patterns using commercial software (Nuance software, CRi, Inc., Woburn, MA). Wavelength-specific images unmixed for RFP and trastuzumab-

RhodG demonstrated that mesenteric tumor implants from individual mice consisted of both SHIN3-RFP and SKOV3 bound-to- trastuzumab-RhodG (Figure 3).

***In situ* real-time tissue sampling**

A comparison of real-time multicolor optical imaging (approximately 65ms/frame) to multispectral imaging (approximately 30s/frame) revealed the later method to be both sensitive and specific (Figure 3a). Multispectral imaging of tumor nodules was performed to compare nodule characterization with real-time multicolor optical imaging (Figure 3b). Image analysis conducted to compare tumor nodule fluorescence revealed that real-time multicolor optical imaging was also both sensitive ($n=5$, $90\pm7.6\%$) and specific ($n=5$, $90\pm8.2\%$) (Table 1).

***In vivo* real-time image-guided tissue sampling**

Results from experiments evaluating the accuracy of tumor removal based on real-time multicolor optical imaging in an *in vivo* mixed tumor model also revealed the method to be both sensitive and specific. Real-time multicolor optical imaging was used to visualize intra-abdominal tumor implants after i.p. trastuzumab-RhodG and tumor nodules were removed and categorized according to their fluorescence pattern (as described above). Multispectral imaging of tumor nodules was used as the gold-standard (Figure 4). Image analysis revealed that real-time multicolor optical imaging was both sensitive ($n=5$, $96\pm4.0\%$) and specific ($n=5$, $96\pm3.4\%$) (Table 2).

Histologic verification of tumor implants

Histologic evaluation confirmed that all 97 tumor nodules removed during *in situ* and *in vivo* experiments were epithelial carcinoma.

Discussion

The value of fluorescence optical imaging for guiding treatment of cancer is a rapidly evolving technique¹³⁻¹⁴. The use of multiple targeted probes is more likely to yield highly specific results than is the use of a single targeted probe. However, the identification and removal of lesions on the basis of their expression profiles will require multiple distinct optical probes, which necessitates the ability to discern different fluorescence signals in real-time. Currently, multispectral imaging is the most sensitive tool available in optical imaging for distinguishing between different fluorescence signals¹⁵⁻¹⁶. Current-generation multispectral cameras are equipped with a tunable crystal filter, which enables the continuous acquisition of data over the entire light spectrum. However, this process takes at least 5 second per frame and therefore, is not amenable to surgical or biopsy procedures that require real time guidance.

This next step forward in molecular imaging will rely on characterizing tissue *in vivo*, which, in turn, depends on the ability to perform multicolor imaging simultaneously. With this in mind, we conducted a series of experiments to evaluate the feasibility of identifying lesions based on color pattern *in vivo* and *in situ*. In these experiments, we evaluated the accuracy of tumor removal based on real-time multicolor optical imaging *in situ* and *in vivo* and demonstrated that this method is both sensitive and specific.

To perform this work we needed to develop a novel coincident tumor model in which mice possessed tumor implants from two different epithelial ovarian cancer cell lines: 1) SHIN3-RFP, which demonstrated red fluorescence due to an RFP-expressing plasmid; and 2) SKOV3, which possessed a distinct molecular phenotype, overexpressing epidermal growth factor receptor, HER2, which we selectively targeted with trastuzumab conjugated to the

fluorophore, RhodG. This model permitted characterization with high sensitivity and specificity of implants with HER2+ cell surface markers based on their fluorescence pattern. The ability of real-time multicolor optical imaging to guide effective resection in live animals suggests that this technology could potentially be used during surgery to improve the visualization and characterization of tumor implants. In this study, we used the HER2 surface molecule as a target because we had access to 2 excellent ovarian cancer models with and without HER2 expression and therefore these cell lines were chosen for the validation of this methodology. In order to apply this technology to the clinical practice, CA125 20 or mesothelin, 21 may be a useful clinical target for imaging ovarian cancer.

Animal tumor models are integral to the development of optical imaging technologies and much progress has been made towards the simulation of human cancers in animals 17-19. However, typical murine tumor models differ from human disease many ways—one of these is that models often lack the heterogeneity commonly encountered in the clinical setting. In humans, various non-cancerous diseases, such as inflammatory nodules, frequently exist within the tumor environment. Using conventional single tumor mouse models to simulate human disease it is possible to obtain true-positive and false-negative tumors but not true-negative and false-positive tumors within the same mice. The coincident tumor model provided a useful tool for the evaluation of antibody-based probes to selectively bind tissue based on surface receptor expression. The differential binding of trastuzumab-RhodG was therefore attributed to the expression of HER2 on the SKOV3 cell line which was absent on the SHIN3-RFP cell line.

The identification of cancerous lesions by distinct receptor profiles will require highly specific probes with minimal nonspecific binding so that molecular phenotypes can be accurately depicted by probe fluorescence. Such specificity becomes increasingly critical as the number of surface molecule targets increases for each cell type. This technology could facilitate the successful biopsy of tumors expressing specific target receptors and can lead to more accurate histological diagnoses in real-time.

Conclusion

A novel two-tumor coincident mouse model of ovarian cancer was established, in which animals simultaneously bore tumor implants with distinct profiles of surface markers to demonstrate that real-time optical imaging permits accurate characterization of tissues possessing more than one cell type and that probe color can be used as an accurate marker to guide the real time removal of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *In vitro* validation of targeting 2 ovarian cancer cell lines with different fluorescence signals

A. Flow cytometry data confirming the presence of cell populations with distinct fluorescence, SHIN3-RFP and SKOV3. B. Fluorescence microscopy data confirming the presence of 2 cell populations with distinct fluorescence characteristics. Fluorescence microscopy utilizing filters specific for red and green wavelengths, confirmed the presence of 2 distinct cell populations. The red fluorescence is secondary to RFP expression from the SHIN3-RFP cell line, while the green fluorescence is due to trastuzumab-RhodG binding HER2 receptors of the SKOV3 cell line.

Figure 2. Establishment of a coincident ovarian cancer model in mice for multicolor optical molecular imaging

Fluorescence microscopy verifying the presence of both SHIN3-RFP and SKOV3 derived peritoneal tumors within the mesentery of the coincident tumor model mice treated with i.p. trastuzumab-RhodG. Fluorescence microscopy permitted visualization of red fluorescence signal from the SHIN3-RFP tumors as well as green fluorescence from trastuzumab-RhodG binding to HER2 receptors of SKOV3 tumor implants and therefore confirmed the ability of the dual injection method to establish a coincident tumor model.

Figure 3.

Loops of bowel with mesenteric tumor implants (A). Loops of bowel with mesenteric tumor implants were imaged with real time multicolor imaging (left) and multispectral imaging (right). SHIN3-RFP tumor implants demonstrated red fluorescence (arrows), while SKOV3 tumor implants demonstrated green fluorescence (arrowheads) from trastuzumab-RhodG binding to HER2 receptors. Asterisks (*) indicate the intestinal loop. Multispectral images of mesenteric tumor implants resected using real time multicolor imaging as a guide (B). Using real time multicolor imaging, nodules were surgically removed and organized according to fluorescence pattern. All nodules demonstrating any green fluorescence (with or without red fluorescence) as seen with real time multicolor imaging were placed in the right column. All nodules demonstrating only red fluorescence were placed in the left column. Multispectral imaging was then used to evaluate the accuracy of tissue fluorescence characterization obtained with real time multicolor imaging (also see Table 1).

Figure 4. Multispectral images of *in vivo* mesenteric tumor implants

Multispectral images of *in vivo* mesenteric tumor implants, which were resected with real-time multicolor image-guided surgery from the abdominal space in 5 coincident tumor bearing living mice (see supplementary video 2). Wavelength-specific images unmixed for RFP and trastuzumab-RhodG demonstrated that mesenteric tumor implants from individual mice consisted of both SHIN3-RFP and SKOV3 bound-to- trastuzumab-RhodG (see also Table 2).

Table 1

Sensitivity and specificity for the detection of HER2+ peritoneal tumors as determined by real-time multicolor optical molecular imaging of mesenteric tumor implants in loops of bowel from five mice ex vivo mice (M1-M5).

	Sens/ Spec (%)	True Positive	True Negative	False Positive	False Negative
M1	75/100	6/6	5/7	0/6	2/7
M2	66/66	2/4	4/5	2/4	1/5
M3	100/100	9/9	2/2	0/9	0/2
M4	100/83	3/4	5/5	1/4	0/5
M5	100/100	6/6	5/5	0/6	0/5
Total	90/90				

Sensitivity and specificity for the detection of HER2+ peritoneal tumors in five live mice models (M6-M10) as determined by *in vivo* real-time multicolor optical molecular imaging.

Table 2

	Sens/ Spec (%)	True Positive	True Negative	False Positive	False Negative
M6	100/100	5/5	5/5	0/5	0/5
M7	100/100	4/4	4/4	0/4	0/4
M8	100/100	6/6	2/2	0/6	0/2
M9	80/100	4/4	3/4	0/4	1/4
M10	100/83	4/5	5/5	1/5	0/5
Total	96/96				