High-throughput single-RNA molecule imaging analysis reveals spatial information of heterogeneous cardiomyocytes expressing *Myh7* RNA in heart failure

（心筋細胞の胎児型遺伝子発現のばらつきと心不全進展の関連性の検討）

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ABSTRACT

Background and Purpose: *Myh7*, beta-type myosin heavy chain, is abundantly expressed in the fetal hearts and soon after birth, expression of *Myh7* are decreased to undetectable level in murine hearts. Although it is well known that pressure overload induces reexpression of fetal gene including *Myh7*, the precise expression patterns remain elusive.

The purpose of the present study is to visualize *Myh7* expressing cells at the single cell level using a novel method.

Methods: We imposed a pressure overload to murine hearts by performing transverse aortic constriction (TAC), which induced adaptive left ventricular hypertrophy (LVH) at 2-week post surgery, followed by heart failure (HF) at 8-week. The sectioned hearts from sham, LVH and HF, were used for single-molecule *in situ* hybridization (smFISH) for *Myh7 mRNA* and were analyzed by automated high-throughput image analysis unit.

Results: Quantitative analysis of *Myh7* mRNA dots per unit area demonstrated that the most abundant expression of *Myh7* gene was found in heart failure heart section, followed in order by hypertrophy and sham sections. *Myh7* gene were observed heterogeneously expressed within cardiomyocytes and localized more abundantly in the middle layer of the hearts.

Conclusions: We have established an automated high-throughput non-biased smFISH analysis at the single cell level and revealed that pressure overload recalls the fetal gene program heterogeneously at cellular and spatial levels in the heart.

KEYWORDS: Heart failure, single cell analysis, RNA *in situ* hybridization, automated high-throughput non bias analysis

1. INTRODUCTION

Heart failure is the leading cause of adult death worldwide, manifested as severe cardiac contractile dysfunction[1,2]. Historically, it was thought to be the end-result that is caused by all the cardiac disorders. However, the mechanism by which failing heart develops remains unclear.

The four-chambered heart consists of different cell types (cardiomyocytes, fibroblast, endothelial cells, immune cells and other types of cell). Among them, cardiomyocytes play major roles in cardiac contractile functions. Cardiac wall stress has been reputed to trigger hearts to undergo hypertrophy eventually leading to cardiac dysfunction with different expressions of many genes[3].

A myosin heavy chain is the molecular motor in cardiomyocytes with two isoforms, *Myh6* and *Myh7*, in mammalian heart [4]. Adult cardiomyocytes in mice primarily express *Myh6*, whereas embryonic cardiomyocytes express *Myh7*[5]. Pressure overload induces reexpression of *Myh7* as the "fetal gene reprograming".

Since the ATPase activity of MYH6 is higher than MYH7[6], the isoform change from MYH6 to MYH7 is considered to be associated with the cardiac dysfunction[7].

To get insights into the mechanism of heart failure, we have examined precise expression level and pattern of *Myh7* after pressure overload in mice hearts, using a

novel method. We established the smFISH on intact heart sections, which is highly sensitive technology with essentially no background, and the automated non-biased histological analysis using In Cell Analyzer 6000, which is a highly sensitive, laser-based confocal imaging platform for high-throughput image analysis[8,9].

Our new novel pipeline firstly revealed heterogeneous expression of *Myh7* in pressure-overload induced failing hearts.

2.METHODS

2.1. Ethical approval: All animal studies were approved by the University of Tokyo Ethics Committee for Animal experiments.

2.2 Mouse model

2.2.1 TAC mouse: We induced cardiac hypertrophy and dysfunction by performing severe transverse aorta constriction (TAC) using a procedure described previously[10]. In brief, the aortic arch was ligated on a 27-gause needle using 7-0 silk suture, and the needle was released causing a constriction of the aorta. In this model, cardiac hypertrophy gradually developed, reached a peak on day 14 after TAC and decreased

afterwards with cardiac systolic dysfunction. Sham-operated animals, which underwent a similar surgical procedure without aortic constriction, were used as controls.

2.2.2 Isolation of single cardiomyocyte from adult hearts: Cardiomyocytes were isolated from the mice hearts using the Langendorff method, as described previously[11]. Briefly, adult cardiomyocytes were isolated by removal of beating hearts from deeply anesthetized mice and cannulated retrograde perfusion with cell isolation buffer (NaCl 130 mM, KCl 5.4 mM, $MgCl_2$ 0.5 mM, NaH2PO4 0.33 mM, D-Glucose 22 mM, HEPES 25 mM, 2,3-Butanedione monoxime 30 mM, pH 7.40) supplemented with collagenase type 2 (Worthington) and protease from Streptomyces griseus Type XIV (Sigma Aldrich). Dissociated cardiomyocytes were singly collected by pipetting up with P2 micropipette under dissection microscope.

2.2.3 Single-cell quantitative PCR: Singly collected cardiomyocytes were deposited into lysis buffer preloaded in a 96 well plate and single cell cDNA synthesis was performed by using Smart-seq2 protocol as previously described[12]. cDNA samples were quantified by quantitative PCR (qPCR) by SYBR Green PCR Kit (QIAGEN, Venlo, Netherlands) on the CFX96 Touch™ (Bio-Rad, Hercules, Calif, USA). The PCR primers used were as follows:

Cox6a2, the forward primer (5'- CGTAGCCCTCTGCTCCCTTA -3') and reverse primer (5'- GGATGCGGAGGTGGTGATAC -3');

Myh7, the forward primer (5'- AATCCGGAGCTGGGAAGACT -3') and reverse primer (5'- GGGTCTGGTCCTTCTTGCTG -3');

Tnnt2, the forward primer (5'- TCCTGGCAGAGAGGAGGAAG -3') and reverse primer (5'- TGCAGGTCGAACTTCTCAGC -3').

The thermal cycling conditions were 3 minutes at 94°C, followed by 42 cycles of 20 seconds at 94°C for denaturing, 20 seconds at 58°C for annealing, and 25 seconds at 72°C for extension. The limit of detection of qPCR value was determined as 40 threshold cycles (Ct). Each sample was consequently normalized to delta Ct to using the expression of *cox6a2*.[13]

2.2.4 Tissue processing: Mice hearts were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm thickness, and used for immunohistochemistry. Hearts were harvested, embedded in Optimal cutting tempreture (OCT compound), sectioned at 10 µm thickness, and used for smFISH.

2.2.5 Immunohistochemistry: Sections were deparaffinized, rehydrated and washed in distilled water. Epitopes were retrieved by heating EDTA buffer (Dako Agilent Pathology solutions) for 20 minutes in a microwave. The sections were blocked in

M.O.M Kit (Vector Laboratories) containing 5% normal goat serum for 1 hour at 4°C, rinsed with phosphate-buffered saline (PBS) for 5 minutes three times. For immunofluorescence, sections were incubated with the following primary antibody overnight at 4 °C; mouse monoclonal anti-MYH7 antibody (1:100 dilution, M8421 SIGMA). Secondary antibody (Alexa Fluoro 546-conjugated anti-mouse monoclonal IgG antibody) and fluorescein conjugated wheat germ agglutinin (WGA; 1:100) were applied to visualize expression of MYH7 protein and cell membrane, respectively. Sections were mounted with ProLong Gold Antifade with DAPI (Life Technologies, Inc.)

2.2.6 Single-molecule fluorescent *in situ* hybridization (smFISH): smFISH was performed with RNAscope® (Advanced Cell Diagnostics (ACD)[8] under manufacturer's instruction. We used a probe against mouse myosin heavy polypeptide 7 (Myh7, NM_080728.2, bp 2-6054, ACD#454741).

For the manual multi-plex RNAscope assays, frozen sections (10 µm) were fixed in PBS containing 4% paraformaldehyde for 15 minutes at 4 °C, and dehydrated by 50%, 70%, and 100% ethanol gradually for 5 minutes at room temperature followed by protease treatment for 30 minutes at room temperature.

Probes were then hybridized for 2 hours at 40 °C followed by RNAscope amplification and costaining with fluorescein conjugated wheat germ agglutinin (WGA; 1:100) to detect cell borders. Sections were counterstained with DAPI.

2.2.7 Image analysis: smFISH images were obtained as Z stacks using Zeiss LSM 510 META (Carl Zeiss GmbH, Oberkochen, Germany), BZ-X710 (Keyence, Osaka, Japan) and In Cell Analyzer 6000 (GE Healthcare UK Ltd). All the images are shown in figures after color conversion between green (488 mm ex. wavelength) and red (594 mm ex. wavelength). Immunohistochemistry was analyzed by BZ Analyzer (Keyence, Osaka, Japan). Dot quantification was performed using In Cell Developer Toolbox (GE Healthcare UK Ltd).

Analytical procedures for the quantitative evaluation of smFISH dots within cardiomyocyte were as follows. Each italic phrase indicates the command operation defined in the software.

Cardiomyocyte Recognition: We selected the channel with the image of the membrane marker (WGA) and *Segmentation* option (intensity segmentation) and set an intensity threshold that identify cell membrane most appropriate for an accurate WGA staining. We defined individual cardiomyocytes reference to membrane marker (WGA) information.

smFISH dot Recognition: We selected the channel with the image of smFISH dot (*Myh7*) and *Segmentation* option (vesicle segmentation) and set an intensity threshold that identify smFISH dots Recognition most appropriate for an accurate *Myh7* mRNA dots.

Counting of smFISH dot within cardiomyocyte: We linked the cardiomyocyte recognition and smFISH dot recognition and made the program which count the smFISH dot only within cardiomyocytes

2.3 Statistical Analyses: Statistical analysis between two groups was determined by the Welch's test. Multiple group comparison was determined by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

3. RESULTS

3.1. Comparison of detection power for *Myh7* expression between single-cell qPCR and immunohistochemistry.

Since the *Myh7* gene expressions have long been recognized as a signature of cardiac hypertrophy and heart failure, we firstly examined the time course of *Myh7* gene expressions with single-cell quantitative PCR analysis (qPCR). Adult cardiomyocytes

were singly isolated from left ventricular free wall using the Langendorff method after 0, 2 and 8 weeks of TAC operation and cDNA syntheses from 132 single-cardiomyocyte using SMART-seq2 protocol[11,12]. Three time points for tissue harvest represent normal (sham), hypertrophied (LVH) and failing heart (HF), respectively. We examined the gene expression levels of the well-established general reference marker, *Cox6a2*[14],and cardiac reference marker *Tnnt2*[15], and confirmed that expression levels of these genes were uniform and stable throughout the time course (data not shown). These results indicated that single cell mRNA was successfully collected, converted into cDNA and correctly amplified to mirror the naïve status of gene profiles of individual cells *in situ*. *Myh7* gene expression was normalized by comparison with expression of the *Cox6a2*. We next explored the expression of *Myh7*.

In the sham heart, virtually no cardiomyocyte expressed *Myh7* mRNA (Fig.1A). In contrast, the proportion of cardiomyocytes expressing *Myh7* mRNA (delta Ct > 10) was increased to 18.7% (9 cells / 47 cells counted) and 42.5% (20 cells / 48 cells counted) in LVH and HF, respectively (Fig.1A). Interestingly, expression levels of *Myh7* mRNA were not homogeneous, and there were many *Myh7*-negative cells in the HF, suggesting that there is the heterogeneity among cardiomyocytes in terms of fetal-gene expression after pressure overload. To investigate the spatial heterogeneity in the heart, we

performed immunohistochemistry with antibodies against Myh7 on the heart section of sham and 4 weeks after surgery, (Fig.1B and 1C). Consistent with single-cell qPCR, there was virtually no MYH7 protein expressions in the sham heart (data not shown). In the 4 week-TAC heart, the strongly MYH7 positive cells were observed in some cardiomyocytes (Fig. 1C), but the distribution of these cells are sparse (Fig. 1B) and positive cells were much less (0.1%) than that observed in qPCR (18% ~ 42%) (Fig. 1A and 1D). The detection sensitivity of single-cell qPCR might be much greater than that of immunohistochemistry and the latter might only detect the cells with delta Ct > 20 or more for *Myh7* mRNA.

3.2 smFISH as a useful tool for analyzing small amounts of molecules in the heart

Single-molecule *in situ* hybridization (smFISH) was firstly reported by Raji et al. 2008[16] to circumvent the difficulty of conventional RNA *in situ* hybridization in gaining single molecule resolution of transcript detection. We tested it in mouse heart frozen sections to detect *Myh7* mRNA with wheat-germ-agglutinin (WGA) membrane staining to delineate cell borders in green (Fig. 2A-2C). Red dots represent *Myh7* mRNA molecule under confocal microscopy. Cardiomyocytes expressing *Myh7* molecules seem to be elevated in hypertrophy (Fig.2B) and heart failure (Fig. 2C) mouse heart compared to

sham mouse heart (Fig. 2A). The density of mRNA dots per cell was visually higher and the number of cells expressing these dots was more in HF section, recapitulating the results of single-cell qPCR. We firstly attempted the quantitative analysis for the number of these molecules, however, it was difficult to manually count all of them under the super high detection power for each molecules of smFISH.

3.3. Automated high-throughput quantification of *Myh7* mRNA

We then developed a pipeline to enable the high-throughput analysis of smFISH data using a highly sensitive, laser-based confocal imaging platform, IN Cell Analyzer, equipped with In Cell Developer Toolbox.

We developed an automated high-throughput quantification program to count *Myh7* mRNA molecule dots in the heart. To avoid the biased observation or analysis, 49 (7x7) fields of view centering at the left ventricular (LV) chamber, were set and analyzed (Fig. 4A). Referring to WGA staining as cell borders in green, approximately 1000 cardiomyocytes were automatically captured and analyzed to measure the cardiomyocyte area, concurrently with the number of *Myh7* mRNA dots within single cardiomyocytes shown in red. (Fig. 3A, 3B). The number of *Myh7* mRNA dots per unit area in the heart sections was measured and calculated using this pipeline. The largest

number of *Myh7* mRNA dots was observed in the heart failure heart section, followed in order by hypertrophy and sham (Fig. 3C). To validate the reliability of this pipeline, we repeated three times and found that results are reproducible and reliable (Fig. 3C). Comparing two histograms from smFISH (Fig. 3C) and single-cell qPCR (Fig. 1A), the distribution of *Myh7* expression profile was similar with each other, further proving the reliability of this newly developed pipeline.

3.4. Heterogeneous expression of *Myh7* in failing heart.

We partitioned the left ventricles into three layers (inner, middle, and outer layer) and analyzed the spatial expression patterns of *Myh7* with smFISH (Fig. 4A). In sham heart, there were few *Myh7* dots in any layers and no difference in the expression patterns among them (Fig. 4B). In contrast, there was a marked increase of *Myh7* dots in all three layers in HF section and the expression patterns between three layers were similar (Fig. 4D). These data suggest that although cellular heterogeneity within each layer is still present, the spatial heterogeneity of *Myh7* positive failing cardiomyocytes occurred at a same degree regardless of the cell location in the heart. In the LVH section, however, an increase of the number of *Myh7* dots was noticeable only in the middle layer (Fig. 4C), indicating that there was heterogeneity of cardiomyocytes between

layers in the hypertrophic stage. These results indicate that individual cardiomyocytes respond to homogeneous pressure stress differentially and suggest that the middle layer of the hypertrophic heart might be most sensitive to pressure overload.

4. DISCUSSION

In the present study, we revealed that cardiomyocytes expressed *Myh7* heterogeneously after pressure overload using a novel high-throughput analysis of single molecule RNA. This novel pipeline has three advantages. First, our method can analyze intact mammalian tissues and has a definite criterion for the positivity of the smFISH dot based on the dot intensity.

Second, we firstly applied smFISH analysis to immunofluorescence staining on heart section. Using high-throughput automated machine (In Cell Analyzer6000), we could achieve further information from the tissue since it has high sensitivity gene expression detection tool and the continuous variable. On the other hand, immunohistochemistry analysis contain the two variables, the positivity and the negativity.

Finally, we could obtain the spatial information of cardiomyocytes expressing *Myh7* in the heart section. Our results show *Myh7* reactivation in cardiomyocytes was not homogeneous phenomenon. We unveiled a part of mechanisms in which individual

cardiomyocytes undergoing homogeneous pressure stress respond differentially and heterogeneously with each other. Besides, heterogeneous reactivation of fetal gene was observed at the level of layer of the left ventricles with abundant *Myh7* expression in middle layer of the hypertrophic heart.

This might be due to the largest wall stress. It was reported that middle layer is wrapped circumferentially[17], which plays major role in cardiac contraction.

It needs further investigation to elucidate the mechanism of cardiac dysfunction by the heterogeneous cardiomyocytes expressing *Myh7*.

The automated high-throughput single molecule RNA analysis we have developed can be applied to many fields of research.

5. CONCLUSIONS

We performed single molecule smFISH and analyzed heart sections using automated high-throughput analysis at the single cell level. The heterogeneity of cardiomyocytes expressing *Myh7* seems to be contributed to the deterioration of cardiac function.

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FIGURE LEGEND

Figure 1. The comparison of the detection power between the single cell qPCR and the immunohistochemistry for MYH7 in the heart.

A: Single cell qPCR of *Myh7* mRNA in cardiomyocytes from sham (N=47), left ventricular hypertrophy (N=48) and heart failure mice (N=47). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

B: Immunohistochemistry on a heart failure mouse (4 weeks after TAC procedure) heart section. MYH7 and DAPI are presented in red and blue, respectively. Scale bar: 200 µm.

C: Magnified view of the boxed region in B. Immunohistochemistry on a heart failure mouse (4 weeks after TAC procedure) heart section. MYH7 and WGA are presented in red and green, respectively. Scale bar: 20 µm.

D: Quantification of the MYH7 positive area/whole cross sectional heart area ratio. $^{\#}p<0.05$ analyzed by the Welch's test compared to sham mice (N=4).

Figure 2. Single molecule fluorescent *in situ* hybridization (smFISH) for *Myh7* in the development of heart failure.

smFISH was performed on a sham (Fig. 2A), left ventricular hypertrophy (Fig. 2B) and heart failure (Fig. 2C) mouse heart section. Scale bar: 20 μm

Figure 3. Automated high-throughput quantification for cardiomyocytes expressing *Myh7* mRNA using In Cell Analyzer 6000.

A: A representative image on a heart failure heart section. The green lines indicates WGA identification of the single cardiomyocyte. Red circles indicates *Myh7* RNA dot identifications within single cardiomyocytes. A merged image is shown in the rightmost column. Scale bar: 20 μm

B: A magnified image of the Fig. 3B. Individual *Myh7* mRNA molecules are well distinguished each other and captured automatically following newly developed algorithm. A merge image is the rightmost. Scale bar: 20 μm

C: The largest number of *Myh7* mRNA dots per unit area (UA) is heart failure heart section, followed in order by hypertrophy and sham. The same trend results were obtained from each experiment. Three independent experiment were performed.

Experiment 1. Cardiomyocytes expressing *Myh7* from sham (N=1005), left ventricular hypertrophy (N=1308), and heart failure mice (N=935). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

Experiment 2. Cardiomyocytes expressing *Myh7* from sham (N=925), left ventricular hypertrophy (N=1303), and heart failure mice (N=763). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

Experiment 3. Cardiomyocytes expressing *Myh7* from sham (N=868), left ventricular hypertrophy (N=1230), and heart failure mice (N=609). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

Figure 4. The reliability and utility of smFISH method to identify the variability of falling cardiomyocytes in spatial and temporal manner

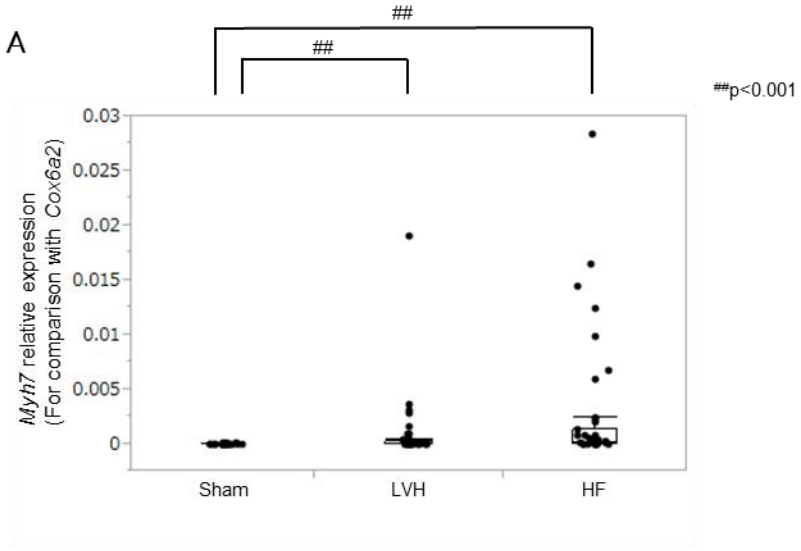
A: A cartoon depicting the location of 49 fields of views acquired on heart section.

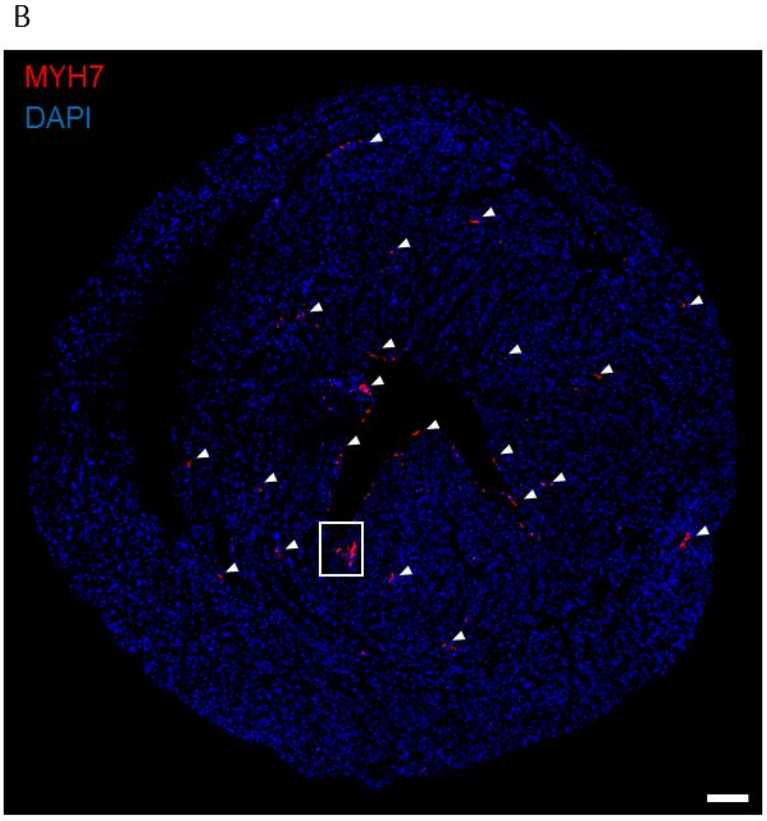
B: sham. Cardiomyocytes expressing *Myh7* from inner layer (N=118), middle layer (N=263), and outer layer (N=487). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

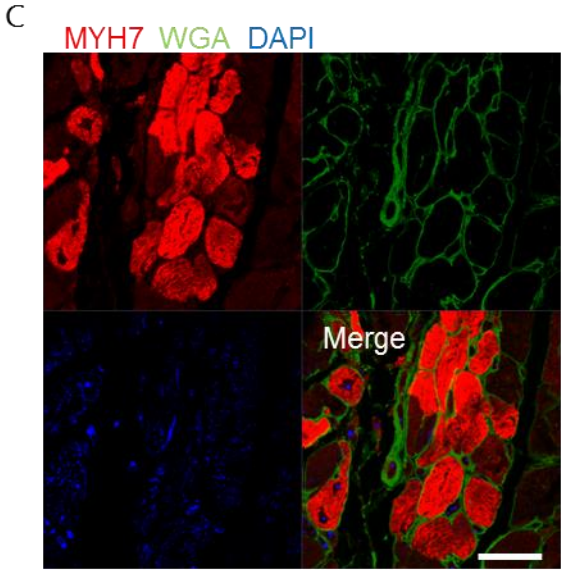
C: LVH. Cardiomyocytes expressing *Myh7* from inner layer (N=263), middle layer (N=519), and outer layer (N=498). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

D: HF. Cardiomyocytes expressing *Myh7* from inner layer (N=141), middle layer (N=261), and outer layer (N=533). $^{\#\#}p<0.001$ analyzed by Kruskal-Wallis tests followed by the Steel-Dwass test for comparison of mean values.

Figure.1 The Comparison of the detection power between the single cell qPCR and the immunohistochemistry for MYH7 in the heart.







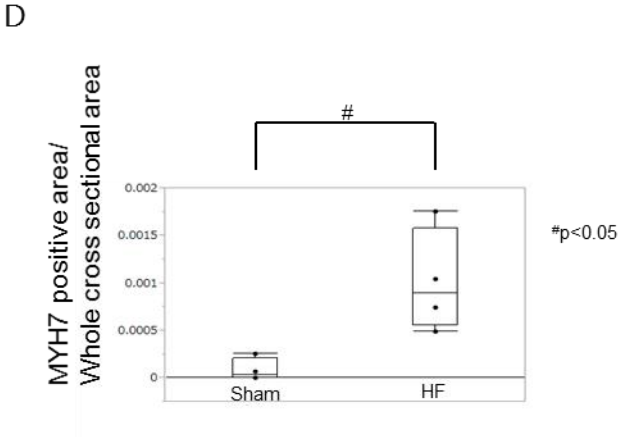


Figure.2 Single molecule fluorescent *in situ* hybridization (smFISH) for *Myh7* in the development of heart failure.

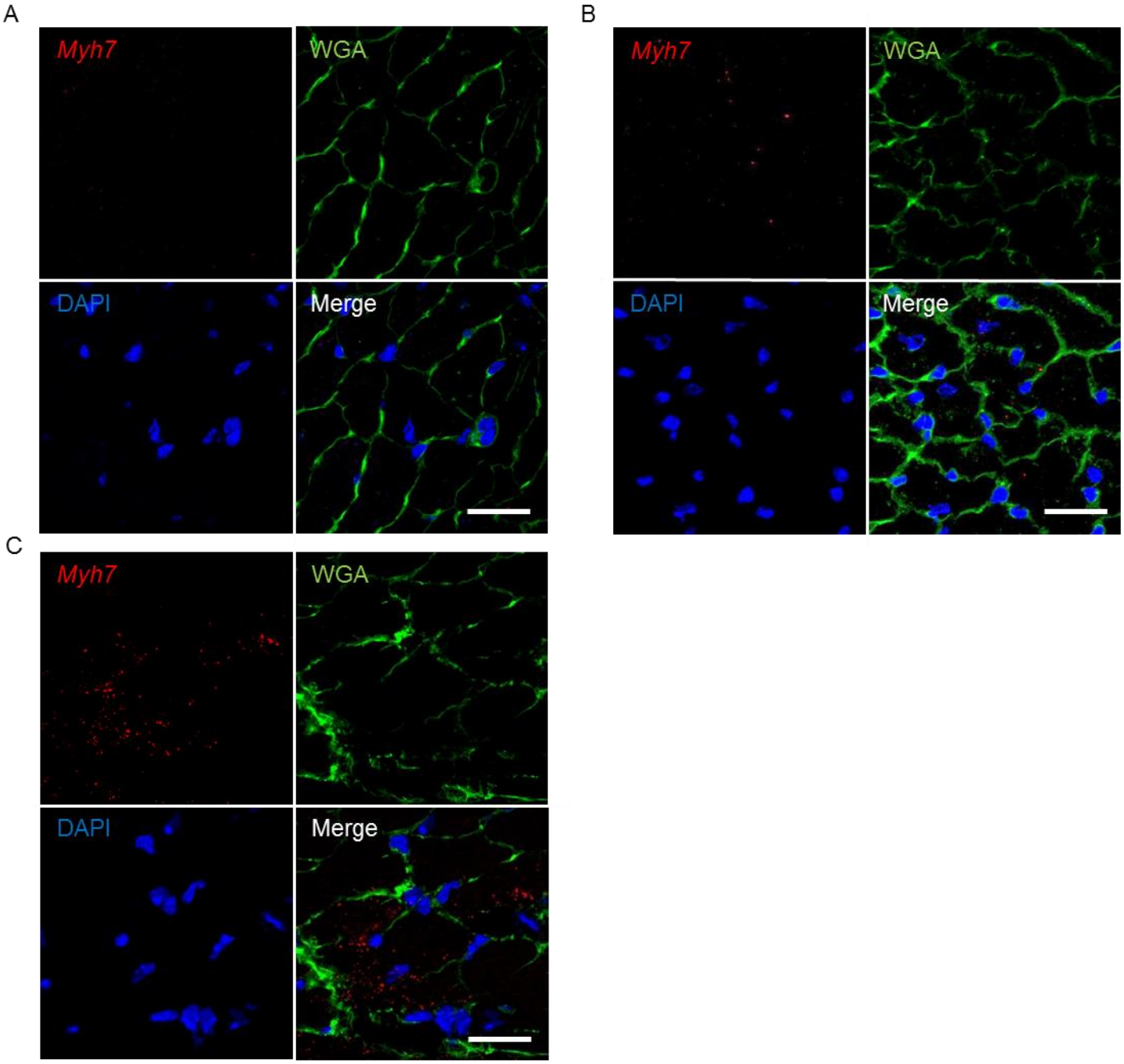
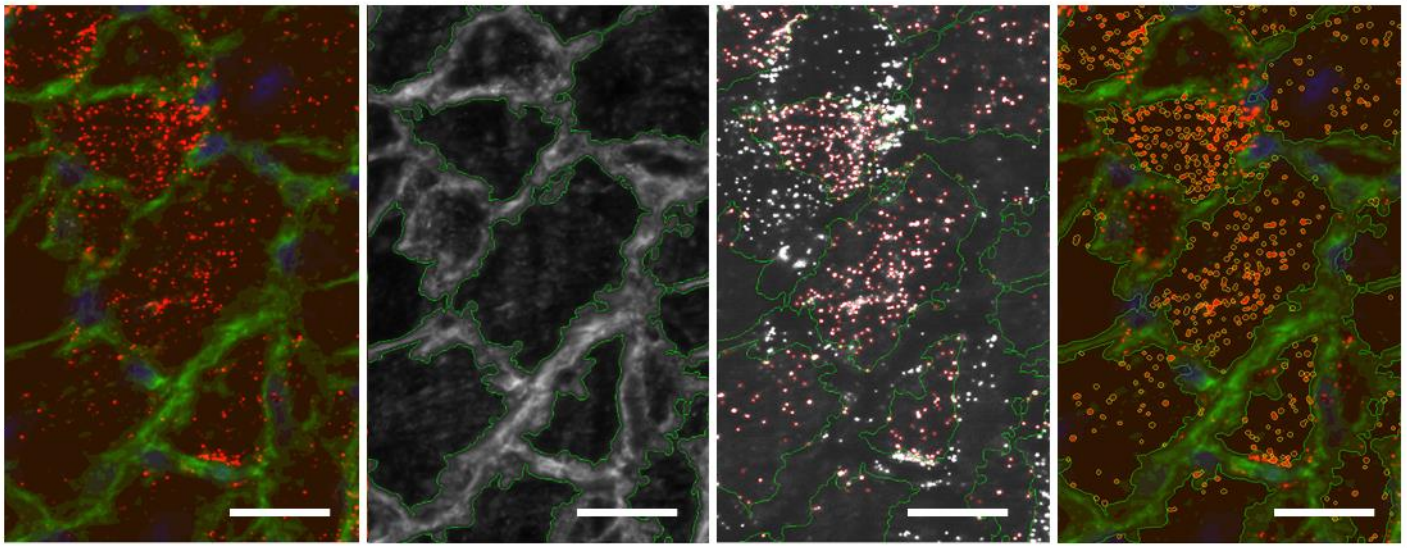


Figure.3 Automated high-throughput quantification for cardiomyocytes expressing *Myh7* mRNA using In Cell Analyzer 6000.

A



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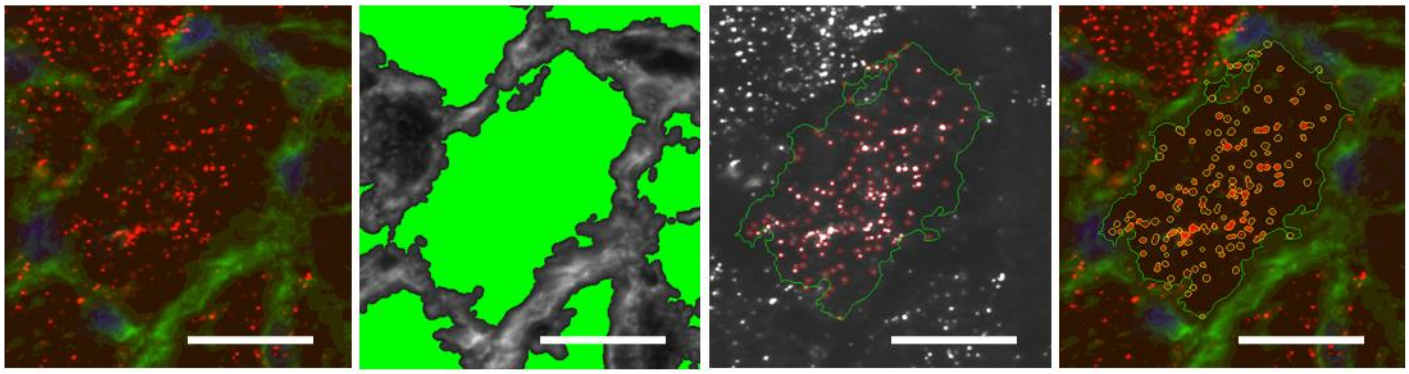

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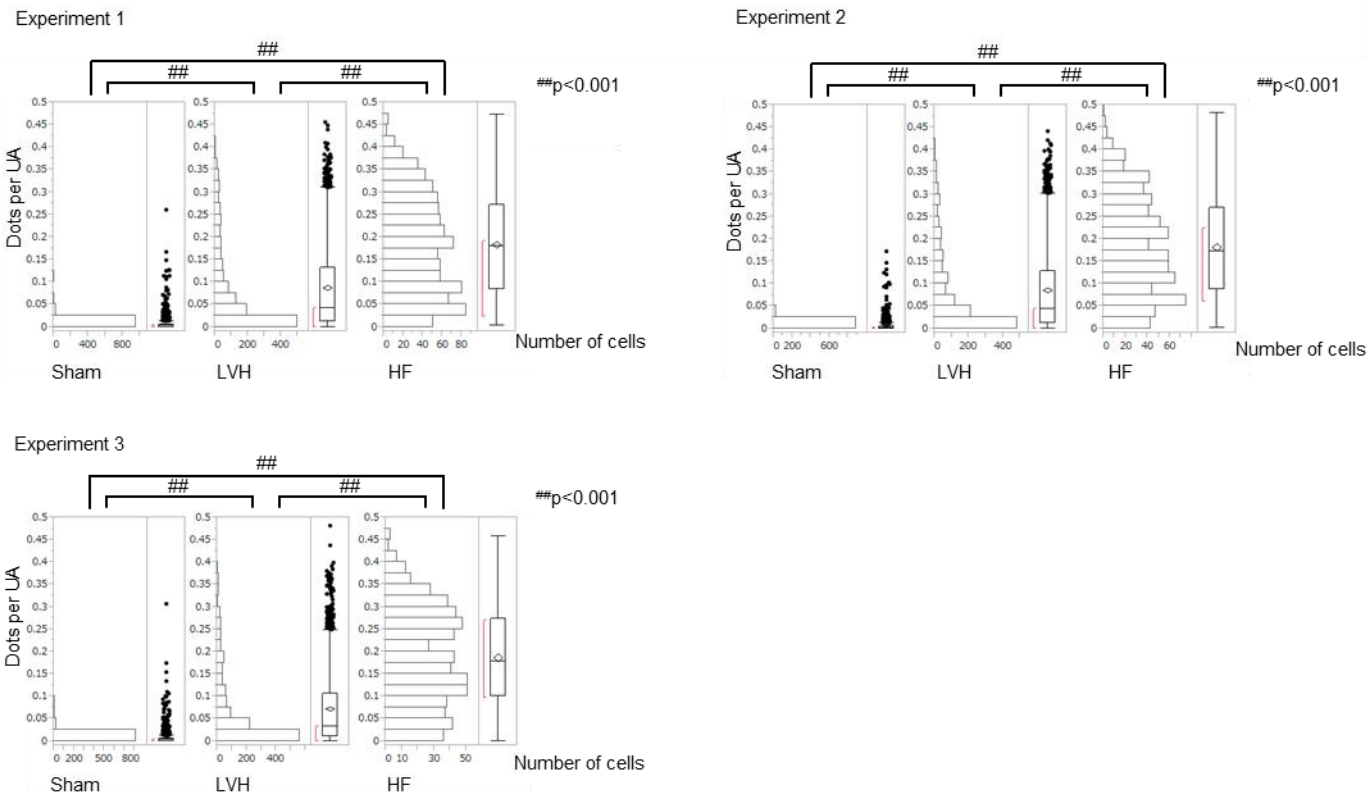
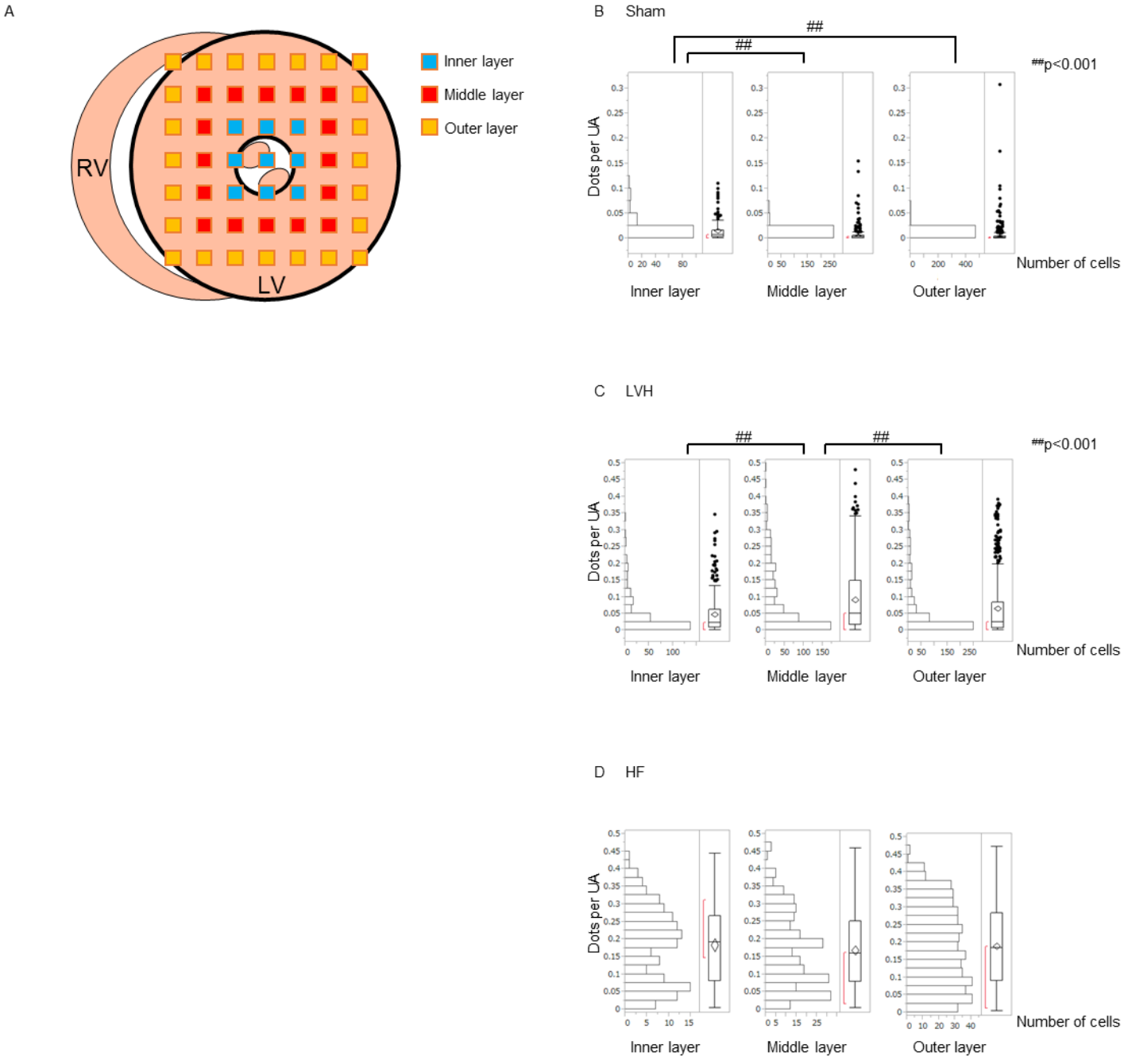


Figure.4 The reliability and utility of smFISH method to identify the variability of falling cardiomyocyte in spatial and temporal manner



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