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Discrimination of DNA mismatches by direct force measurement for identification of tuna species

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Abstract

The interaction between oligonucleotides and long DNA fragments was analyzed by force curve measurements using atomic force microscopy (AFM). DNA fragments (150-base or 406-base) from the mitochondrial ATPase and cytochrome oxidase subunit III genes that contained a mismatch of one to three bases among *Tunnus* species were immobilized on glass slides. The statistical distribution of disruption forces between oligonucleotide probes (21-mer or 29-mer) and single stranded DNA fragments (150-base or 406-base) were analyzed by 40 or 180 force curve measurements. Histograms plotting the frequencies of disruption forces showed a wide distribution with a highest peak. The highest mean values in disruption force were obtained when DNA fragments with perfectly match sequences were employed. These results demonstrated that the specific sequence differences between long DNA fragments can be measured using force-based detection. A single base mismatch yielded a statistically significant 10% decrease in disruption force, furthermore, 2-base and 3-base mismatches provided approximately 20 and 25–30% decreases, respectively. Our results indicated that force-based detection potentially can be applied toward many other mismatched DNA detection techniques besides species-specific identification of tuna.

Keywords: Atomic force microscopy; Force curve measurement; Disruption force; Long DNA fragment; Tuna species identification

1. Introduction

The direct measurement of the interaction between biomolecules is one of the most simple and powerful techniques to analyze the molecular recognition of protein, DNA and mRNA without labeled probes. Atomic force microscopy (AFM) cantilever functionalized with biomolecules has been used to measure the interacting forces between streptavidin/biotin [1,2], antigen/antibody [3–6] and complementary oligonucleotide pairs [7–10], respectively. These studies indicated that force-based detection is a promising sensor technology in terms of label-free operation. Furthermore, force-based detection allows rapid assay completion because the biomolecular interactions are measured by a mechanical process, i.e., force–distance curve measurement using AFM.

Force-based measurement works well for distinguishing binding events, such as specific antibody/antigen reactions and hybridization of complementary DNA duplexes, from unbinding events. Conversely, only a little information has been obtained as to whether minute differences, such as interactions between DNA duplexes with and without a 1-base mismatch, can be discriminated. Sattin et al. have reported that the measurement of the disruption forces of single oligonucleotide pairs with single or two bases mismatch by AFM force curve analysis [11]. Recently, newly designed cantilevers that enable discrimination of DNA single-nucleotide mismatches have been reported [12,13]. The cantilevers transduce molecular recognition into mechanical responses, i.e., optical deflection as a function of molecular interactions at the cantilever surface and the specific resonance frequency of the cantilever. However, discrimination of specific sequences in long DNA fragments using force-based detection remains untested although the interaction events between short oligonucleotide pairs have been well studied.

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Table 1
DNA sequences of oligonucleotide probes for discrimination of *Tunnus* species

Probes	Sequences	Target
NR1 site (21-mer)		
NR1a	5′-SH-TTTAATGGCCCTAGCCTTAAC-3′	NBTA
NR1b	5′-SH-TTTAATAGCCCTAGCCTTAAC-3′	SBT, ALB, NBTP
NR1c	5′-SH-TTTAATAGCCCTAGCCCTAAC-3′	BE, YF
13-bases mismatched	5′-SF-GTTGATTAGCGATGTCGTCGT-3′	Control
NR 2 site (29-mer)		
NR2a	5′-SH-ATTGCCACAGTTGGCATTAGTGGTAGAAG-3′	NBTA, SBT, BE
NR2b	5′-SH-ATAGCTACAGTTGGCATTAGTGGTAGAAG-3′	ALB
NR2c	5′-SH-ATTGCTACGGTTGGCATTAGTGGTAGAAG-3′	YF
NR2d	5′-SH-ATAGCTACAGTTGGCATTAGTGGTAGGAG-3′	NBTP

Underline indicates base mismatch.

Previously, we developed a mismatched DNA detection system based on magnetic capture hybridization technology that has been used for the identification of tuna [14] and cyanobacteria [15], and the detection of single-nucleotide polymorphisms (SNPs) in the ALDH2 gene [16]. Here, we focused on the identification of *Tunnus* species as a target for multiple mismatched DNA detection. Exact genetic identification of *Tunnus* species is necessary, particularly for foreign trade, because the northern bluefin tuna is most highly rated as a "sashimi" product for the Japanese market, which consequently has accelerated illegal fishing and trading. The nucleotide sequences of a 914 bp fragment (ATCO) flanking the mitochondrial ATPase and cytochrome oxidase subunit III genes, whose sequences are highly conserved and contain 1-base to 3-base mismatches among six *Tunnus* species [17–19], were used for the identification of tuna species.

Here we demonstrated the species-specific identification of six tuna species using force-based detection. DNA fragments (150 and 406 bp) amplified from the ATCO region in *Tunnus* species and oligonucleotide probes (21-mer and 29-mer) were immobilized onto a gold-coated glass slide and AFM tip, respectively. The interactions between oligonucleotides and long DNA fragments then were analyzed by force curve measurements using a commercial AFM. In addition to the results, the difference in disruption forces between oligonucleotide pairs and oligonucleotide/long DNA fragment is discussed.

2. Experimental

2.1. Species-specific oligonucleotide probes

Table 1 shows the *Tunnus* species-specific oligonucleotide probes used in this study. Seven types of sequences from two sites, NR1 and NR2 in the ATCO region (Fig. 1) were used as probes for the discrimination of six tuna species, i.e., Atlantic northern bluefin tuna (NBTA), *Tunnus thynnus orientails* (gene accession number: AF260431); Pacific northern bluefin tuna (NBTP), *T. thynnus thynnus* (gene accession number: AF115272); yellowfin tuna (YF), *T. albacares* (gene accession number: AF115278); bigeye tuna (BE), *T. obesus* (gene accession number: AF260430); albacore tuna (ALB), *Thunnus alalunga* (gene accession number: AF115275); southern bluefin tuna (SBT), *T. maccoyi* (gene accession number: AF115277). The NR1 and NR2 oligonucleotide probes contained 1–3 nucleotide differences among the species. All oligonucleotides with and without 5′-SH modification used in this experiment were synthesized by Takara Shuzo (Kyoto, Japan).

2.2. PCR amplification of the ATCO gene in the mitochondrial DNA

Tuna mitochondrial DNA was extracted from the muscle tissue of frozen NBTA, NBTP, YF, BE, ALB and SBT using a magnetic bead purification kit (Magextractor-Genome, Toyobo, Japan) and an automatic nucleic acid purification system (SX-6g, Precision System Science, Chiba, Japan). ATCO genes containing an NR1 site (150 bp) or an NR2 site (406 bp) were amplified by PCR using the following primers: NR1-fragment, NR1R: 5′-HS-ATGAATCAGTTTTGAAGGGTT-3′ and L8562: 5′-CTTCGACCAATTTATGAGCCC-3′; NR2-fragment, NR2F: 5′-HS-CAACTAATCGCTACAGCAGCA-3′ and H9432: 5′-GCCATATCGTAGCCCTTTTTG-3′ (Fig. 1). Amplification was performed in a reaction volume of 40 μl containing 2 μl of DNA sample, 0.06 U/μl Taq DNA polymerase, 0.8 mM dNTPs, 2.5 μM primers and 4 μl of 10× PCR buffer. The thermal profile

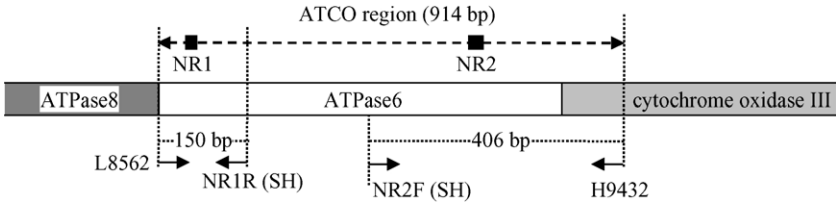

Fig. 1. Schematic diagram of ATCO region flanking the mitochondrial ATPase and cytochrome oxidase subunit III genes.

included an initial denaturation step at 94 °C for 2 min followed by 30 cycles of denaturation at 93 °C for 1 min, primer annealing at 52 °C for 1 min, primer extension at 72 °C for 45 s and a final long extension at 72 °C for 30 min. After purification, the PCR products were used for immobilization on the gold surface.

2.3. Immobilization of thiolated DNA on the gold surface

The oligonucleotide probes with a 5′-SH modification were immobilized on a gold-coated AFM-tip (Si_3N_4, spring constant: 0.11 N/m, frequency: 37 kHz, Seiko Instruments, Japan) as follows: the gold-coated tips were immersed in 100 μM oligonucleotide solution for 2 h at room temperature. After rinsing with ultra pure H_2O, the tips were immersed in 1 mM 6-mercapto-1-hexanol (MCH) for 1 h at room temperature for blocking of non-treated gold surfaces. The oligonucleotides tips were rinsed thoroughly with ultra pure H_2O before use. The immobilization of PCR products with 5′-SH modification onto gold-coated glass slides was performed in the same manner as described above. PCR products were denatured at 95 °C for 10 min and subjected to the immobilization process onto the gold-coated glass slide.

2.4. Force measurements

The disruption forces were measured by force curve measurement using a commercial atomic force microscope (SPI3800, Seiko Instruments, Tokyo, Japan). Force curve measurements were performed in a liquid cell at room temperature. The velocity of the AFM-tip in approach and retraction cycle was constant at 30 nm/s. The measurement of disruption forces between oligonucleotide pairs was conducted in the hybridization buffer (200 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.0). The pH, KCl concentration and 6-mercapto-1-hexanol (MCH) concentration for blocking was optimized to obtain the highest disruption force before data collection. All measurements using oligonucleotides were performed in the same buffer. Average forces were obtained from 40 or 180 force curve measurements. The mean values were calculated as $1/n\Sigma x$ (n: number of measurements, x: disruption forces in each measurement). The NR1a probe (Table 1) was immobilized on the AFM-tip. Three types of thiolated oligonucleotides, which were perfectly matched, 1-base mismatched and 2-base mismatched compared to the NR1a probe, were immobilized onto a gold-coated glass slide. All force curve measurements were performed using the same cantilever.

Species-specific discrimination of *Tunnus* species was performed by a combination of force curve measurements using seven different cantilevers with each oligonucleotide probe in Table 1 (NR1a, NR1b, NR1c, NR2a, NR2b, NR2c and NR2d) and PCR product immobilized onto glass slides. Measurements were conducted in the same manner as described above, except for the concentration of KCl (300 mM) in the hybridization buffer.

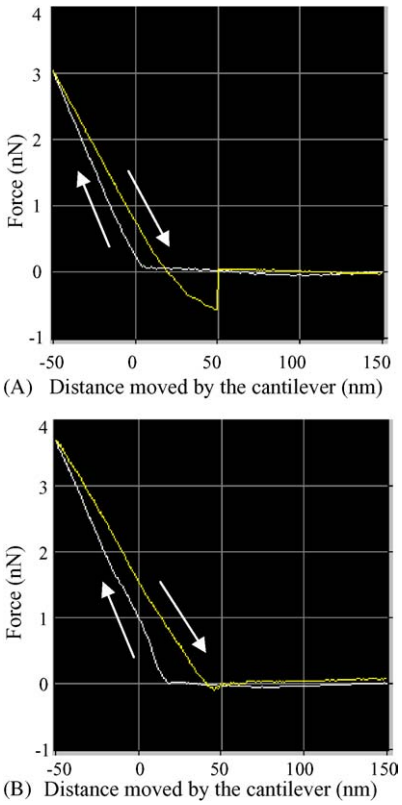

Fig. 2. Force–distance curves on the approach and retraction of the NR1a oligonucleotide-tip on the surface of complementary oligonucleotide (A) and double stranded oligonucleotide (B).

3. Results

3.1. Analysis of disruption forces between oligonucleotide pairs

Fig. 2(A) shows a typical force–distance curve using the NR1a oligonucleotide-tip and the complementary oligonucleotide immobilized on glass slides. A typical jump toward or away from contact was observed. The events were not obtained when double stranded oligonucleotide was used (Fig. 2(B)). These results suggested that the disruption forces were caused by hybridization between oligonucleotide pairs. On the other hand, the disruption forces at less than 85 pN were detected between oligonucleotide and double stranded oligonucleotide with low frequency. Therefore, the data at less than 85 pN were eliminated as negligible interaction events in the following experiments. The statistical distribution of disruption forces between oligonucleotide pairs was analyzed by obtaining 180 force curve measurements when the NR1a oligonucleotide-tip was used. Three different oligonucleotide pairs were studied as targets, i.e., 21-mer oligonucleotides that had complementary sequences to NR1a, NR1b and NR1c, were immobilized onto the glass slide. Histograms plotting the frequencies of disruption forces showed a wide distribution with a highest peak (Fig. 3). The highest peak shifted to lower disruption force with increasing base mismatch. The peak shift was approximately 60 pN (three columns apart) between Fig. 3(A) and (B), or Fig. 3(B)

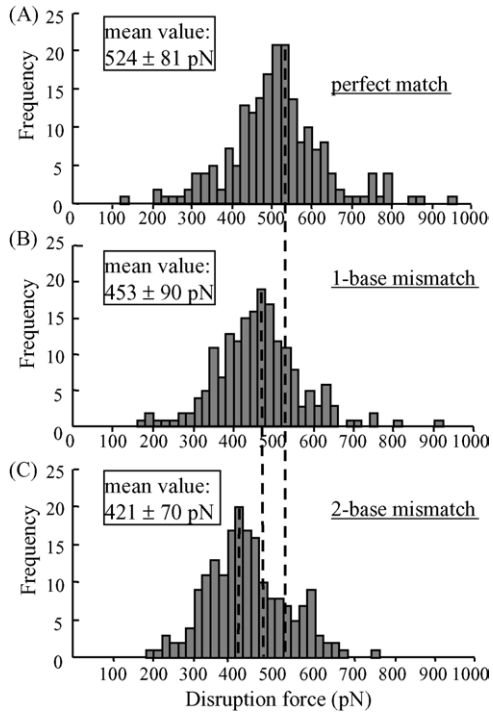

Fig. 3. Histogram of disruption forces between NR1a oligonucleotide-tip and complementary oligonucleotides to NR1a (A), NR1b (B) and NR1c (C) probes immobilized on glass slides.

and (C). The mean values of disruption forces were 524 ± 81 pN for a perfectly matched sequence (NR1a), 453 ± 90 pN for a 1-base mismatched sequence (NR1b) and 421 ± 70 pN for a 2-base mismatched sequence (NR1c). The difference between perfectly matched/1-base mismatched (71 pN) had a greater disruption force than that between 1-base mismatched/2-base mismatched (32 pN). This could be due to the difference between hydrogen bond formation of a GC base pair versus an AT base pair. A GC base pair in the perfect match sequence was replaced with a GT mismatch in NR1b, while a TA base pair was replaced by a TG mismatch in NR1c. These results suggested that force-based detection could allow detection of mismatched DNA at a single base pair level to obtain the highest mean values. At least 40 force curve measurements for each sample were necessary to obtain the histograms.

3.2. Force-based detection of specific sequences in long DNA fragments

To investigate the applicability of force-based detection to long DNA fragments, PCR products were subjected to force curve measurements. Table 2 shows mean values of disruption forces between the NR1a probe (21-mer) and NR1 gene fragments (150-bases), and the NR2a probe (29-mer) and NR2 gene fragments (406-bases). The highest disruption forces were obtained when gene fragments containing perfectly match sequences were used as targets. Furthermore, 1-base, 2-base or 3-base mismatches could be discriminated by the disruption forces. These results demonstrated that the specific sequence in a long DNA fragment could be discriminated by force-based detection. The disruption forces in NR2a were relatively higher than those in NR1a because a longer oligonucleotide (29-mer) in the NR2 site was used for detection.

Table 2
Mean values of disruption forces between NR1a or NR2a oligonucleotide-tip and PCR product-slide

Target PCR products	Disruption force (pN)
NR1a probe (21-mer)	
Perfect match	
NBTA (150-base)	483 ± 53
1-base mismatch	
SBT	437 ± 55
ALB	431 ± 46
NBTP	428 ± 48
2-base mismatch	
BE	391 ± 45
YF	384 ± 48
NR2a probe (29-mer)	
Perfect match	
NBTA (406-base)	525 ± 46
2-base mismatch	
ALB	432 ± 41
YF	420 ± 40
3-base mismatch	
NBTP	398 ± 38

3.3. Identification of tuna species based on force analysis

Multiple combinations of force curve measurements were conducted using 7 types of oligonucleotide probes (Table 1) and 12 different PCR products (NR1 and NR2 fragments in 6 *Tunnus* species) for the identification of tuna. Table 3 shows the relative percentages of the disruption forces between NR1 probes (21-mer) and NR1 fragments (150-base). The highest disruption forces detected in this experiment were defined as 100%. All perfectly matched NR1 probe-PCR products pairs provided 99–100% of the disruption forces. The relative percentages of 1-base mismatches and 2-base mismatches ranged from 87 to 91% and 79 to 82%, respectively. Table 4 shows the relative percentages of the disruption forces between NR2 probes (29-

Table 3
Relative percentage of disruption forces between NR1 oligonucleotide-tips and NR1 fragment-slide

Target	Relative percentage (%)		
	NR1a probe[a]	NR1b probe[b]	NR1c probe[c]
NBTA	**100** (0)	87 (1)	82 (2)
SBT	90 (1)	**99** (0)	91 (1)
ALB	89 (1)	**100** (0)	89 (1)
NBTP	89 (1)	**99** (0)	90 (1)
BE	81 (2)	89 (1)	**99** (0)
YF	79 (2)	90 (1)	**100** (0)

Values in parenthesis indicate the number of base mismatch. Bold letters indicate perfect matched DNA pairs.
[a] NR1a: NBTA.
[b] NR1b: NBTP, SBT and ALB.
[c] NR1c: BEandYF.

Table 4
Relative percentage of disruption forces between NR2 oligonucleotide-tips and NR2 fragment-slide

Target	Relative percentage (%)			
	NR2a probe[a]	NR2b probe[b]	NR2c probe[c]	NR2d probe[d]
NBTA	**99** (0)	83 (2)	79 (2)	72 (3)
SBT	**98** (0)	82 (2)	82 (2)	71 (3)
ALB	82 (2)	**100** (0)	80 (2)	90 (1)
NBTP	75 (3)	91 (1)	71 (3)	**100** (0)
BE	**100** (0)	82 (2)	81 (2)	72 (3)
YF	79 (2)	82 (2)	**100** (0)	74 (3)

Values in parenthesis indicate the number of base mismatch. Bold letters indicate perfect matched DNA pairs.
[a] NR2a: NBTA, SBT and BE.
[b] NR2b: ALB.
[c] NR2c: YF.
[d] NR2d: NBTP.

mer) and NR2 fragments (406-base). All perfectly matched NR probe-PCR product pairs gave 98–100% of disruption forces. The relative percentage was reduced to 79–83% in the 2-base mismatches, and reduced to 71–75% in the 3-base mismatches.

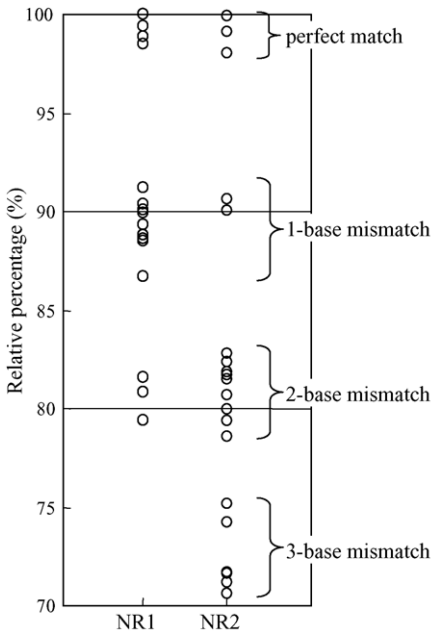

Fig. 4. Distribution of the relative percentage of disruption forces in the NR1 and NR2 sites.

4. Discussion

This is the first report to detect DNA mismatches by force-based detection using actual PCR products. Although DNA mismatches were successfully discriminated using PCR products, where the difference in disruption forces between oligonucleotide pairs and oligonucleotide-long DNA fragment was observed. The mean values of disruption forces between NR1a probe (21-mer) and NR1 fragment (150-base) (483 pN; Table 2) were lower than those between NR1a oligonucleotide pairs (524 pN) (Fig. 3). One possible reason could be that the mishybridizations occurred when PCR products were used as targets. The oligonucleotide probe on tip could bind to non-specific site in PCR products, resulting in lower mean values compared to those between oligonucleotides. The histograms including disruption forces by mishybridization were shifted to the lower side. Furthermore, the difference in the force between perfect match/1-base mismatch was approximately 50 pN when PCR products were used, while the difference between oligonucleotide pairs with the same sequence was 71 pN (Fig. 3). The difference could be originated from error signals introduced by mishybridization. Further studies should be performed to understand the mechanisms involved.

In summary, 1-base mismatch and 2-base mismatch provided approximately 10 and 20% decrease and 3-base mismatch provided approximately 25–30% decrease in disruption force compared to the disruption forces between perfect match oligonucleotide-PCR products pairs (Fig. 4). The disruption forces obtained in this study are possibly derived from multiple disruption events because the number of oligonucleotides within the tip contact area (300 nm^2) could be estimated 3–30 molecules [20]. This might be the possible reason of the larger force value between the oligonucleotides than the previous reports [9,11]. The decrease in disruption force with base mismatch was approximately 40–50 pN, which was independent of the length of the PCR products (150-base or 406-base) and DNA probes (21-mer or 29-mer).

In practical use of force-based DNA detection system, a large number of PCR products spotted on Au substrate should be prepared as samples. Each force curve measurement was completed in approximately 10 s in this study. The use of continuous force analysis system will allow to improve time resolution over DNA chip technology. Recently, a novel force-based sensor using a polymeric anchor (polydimethylsiloxane) in place of the silicone cantilever has been proposed [21,22]. Therefore, force-based detection is relatively portable and potentially could be applied toward the other mismatched DNA detection techniques in addition to species-specific detection of tuna.

5. Conclusions

We demonstrated force-based detection of specific sequences in long DNA fragments. A single base to 3-base mismatch in PCR products (150-base and 406-base) provided a significant reduction of the disruption forces, resulting in species-specific discrimination of six tuna species. Furthermore, force-based detection can be performed at room temperature without rigid temperature control. The force-based detection potentially could be applied toward many other mismatched DNA detection techniques in addition to species-specific detection of tuna.

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